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(54) Title: ANTIBODY WHICH SPECIFICALLY BINDS TO HUMAN CRTH2

(54) 発明の名称: ヒトCRTH2に特異的に結合する抗体

(57) Abstract: The present invention pertains to: an anti-human CRTH2 antibody which specifically recognizes and binds to human CRTH2; a fragment of the antibody; DNA which encodes the amino acid sequence of the antibody; a vector including the DNA; an antibody-producing cell and a hybridoma which produce the antibody; a production method for the antibody; a composition which includes the antibody or antibody fragment; a treatment method and a diagnostic method which use the antibody or antibody fragment for diseases, such as allergenic diseases, autoimmune diseases, diseases associated with eosinophilia and hyperfunction, and diseases associated with an increase in and/or hyperfunction of Th2 cells; and a medicine and a diagnostic agent which include the antibody or antibody fragment.

(57) 要約: 本発明は、ヒトCRTH2を特異的に認識し、結合する抗ヒトCRTH2抗体、該抗体断片、該抗体のアミノ酸配列をコードするDNA、該DNAを含むベクター、該抗体を生産するハイブリドーマおよび抗体生産細胞、該抗体の製造方法、該抗体または抗体断片を含む組成物、該抗体または抗体断片を用いるアレルギー性疾患、自己免疫疾患、好酸球増多や機能亢進を伴う疾患、Th2細胞の増多や機能亢進を伴う疾患などの治療方法および診断方法、並びに該抗体または抗体断片を含む医薬および診断薬に関する。



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明 細 書

発明の名称： ヒトC R T H 2 に特異的に結合する抗体

技術分野

[0001] 本発明は、ヒトC R T H 2 を特異的に認識し、結合する抗ヒトC R T H 2 抗体、該抗体断片、該抗体のアミノ酸配列をコードするDNA、該DNAを含むベクター、該抗体を生産するハイブリドーマおよび抗体生産細胞、該抗体の製造方法、該抗体または抗体断片を含む組成物、該抗体または抗体断片を用いるアレルギー性疾患、自己免疫疾患、好酸球増多や機能亢進を伴う疾患、T h 2 細胞の増多や機能亢進を伴う疾患などの治療方法および診断方法、並びに該抗体または抗体断片を含む医薬および診断薬に関する。

背景技術

[0002] ヒトC R T H 2 (C h e m o a t t r a c t a n t r e c e p t o r - h o m o l o g o u s m o l e c u l e o n T h 2 c e l l s) は、G P R 4 4、C D 2 9 4、D P 2 などの別名でも知られる7回膜貫通型Gタンパク質共役型受容体(G p r o t e i n - c o u p l e d r e c e p t o r、以下、GPCRと記す)であり、プロスタグランジンD2(以下、P G D 2と記す)に対する受容体の一つであることが知られている(非特許文献1)。C R T H 2 は、1996年にヒトT h 2 特異的タンパク質としてクローニングされ、B 1 9と称して開示されている(特許文献1)。

[0003] C R T H 2 は、リガンドであるP G D 2および13, 14-dihydro-15-keto prostaglandin D2(以下、DKPGD2と記す)に代表されるP G D 2代謝物と結合し、細胞内にG α iタンパク質を介したシグナルを伝達し、その結果、C R T H 2 発現細胞の遊走および活性化に関与することが知られている(非特許文献1)。

[0004] ヒトC R T H 2 はT h 2 細胞、好酸球、好塩基球および2型自然リンパ球(Type 2 innate lymphoid cells、以下、I L C 2と記す)などに発現が認められている(非特許文献1、2)。C R T

H2は、Th2サイトカイン産生細胞に特異的に発現する表面マーカーであることが報告されている（非特許文献3）。

[0005] また、ILC2は2011年にヒトにおいて同定されたアレルギー応答に関与する新規細胞集団であり、本細胞を規定する特異的な表面マーカーとしてCRTH2が挙げられている（非特許文献2）。また、nonclassical monocyteやTh2/Th17細胞にCRTH2が発現していることが報告されている（非特許文献4、5）。

[0006] 喘息をはじめとするアレルギー疾患において、CRTH2発現細胞は病態に寄与することが知られている。喘息患者における気管支肺胞洗浄液中の細胞においては、健常人と比較して高頻度でCRTH2陽性T細胞が認められることが報告されており（非特許文献6）、アトピー性皮膚炎においては、重症度と相関してCRTH2陽性T細胞が増加することが報告されている（非特許文献7）。

[0007] 好酸球は細胞傷害性を有する顆粒蛋白質を含んでおり、該蛋白質の沈着が慢性気管支喘息患者の気道組織あるいはアトピー性皮膚炎患者の病変部位に認められることなどから、好酸球は慢性気管支喘息またはアトピー性皮膚炎などのアレルギー性疾患の病態形成において重要な働きをしているものと考えられている（非特許文献8、9）。

[0008] 好塩基球は細胞内にヒスタミンやロイコトリエンといった炎症性分子を貯留し、細胞表面に発現するFcε受容体やFcγ受容体のクロスリンクにより、該分子を放出することにより、アレルギー反応の惹起に関わっている（非特許文献10）。

[0009] ILC2は気道粘膜や皮膚といった局所に存在する細胞であり、組織障害に伴い産生されるインターロイキン（以下、ILと記す）-25、IL-33といったサイトカインに応答し、大量のTh2サイトカインを産生するという特性を持ち、アレルギー疾患の病態形成に関与していると考えられている（非特許文献11）。

[0010] CRTH2に対するモノクローナル抗体として、301108（R&D社

）が市販されている。またBM16が知られている（特許文献2）。これらはげっ歯類抗体であり医薬品としては開発されていない。

[0011] さらに、クローン19A2に関する遺伝子組換えキメラ抗体およびヒト化抗体が、エフェクター活性によりCRTH2発現細胞の除去を行うこと、クローン8B1に関するヒト化抗体やクローン3C12および31A5に関するマウス抗体が、CRTH2に対するアンタゴニスト活性を有することが示されている。

[0012] またクローン19A2に関する抗体はヒトマスト細胞に対しても反応性を有することが示されている（特許文献3）。

先行技術文献

特許文献

[0013] 特許文献1：日本国特許第3144805号公報

特許文献2：国際公開第97／46677号

特許文献3：国際公開第2014／144865号

非特許文献

[0014] 非特許文献1：The Journal of Experimental Medicine, 2001. 193(2):p.255-261.

非特許文献2：Nature Immunology, 2011. 12(11):p.1055-1062.

非特許文献3：European Journal of Immunology, 2000. 30(10):p.2972-2979

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非特許文献4：Blood, 2011. 118(5):e16-31.

非特許文献5：Journal of Allergy and Clinical Immunology, 2014.134(5):p. 1175-1186. e7.

非特許文献6：Clinical & Experimental Immunology, 2010. 161(1):p. 34-40.

非特許文献7：Journal of Investigative Dermatology, 2002. 119(3):p. 609-616.

非特許文献8：Advances in Immunology, 1986. 39:p. 177-253.

非特許文献9 : Immunology Today, 1992. 13(12):p. 501-507.

非特許文献10 : Journal of Allergy and Clinical Immunology, 2013. 132(4):p. 789-801.

非特許文献11 : Journal of Allergy and Clinical Immunology, 2014. 134(3):p. 671-678.

発明の概要

発明が解決しようとする課題

[0015] これまでに複数のヒトC R T H 2抗体が確立されているが、種々のヒト免疫細胞への反応性や、ヒトC R T H 2への特異的結合活性、またはヒトC R T H 2リガンド依存的な活性への影響など、所望の活性を有する抗ヒトC R T H 2抗体の確立が望まれていた。

[0016] 本発明の目的は、ヒトC R T H 2の特徴的なエピトープを認識し、結合することで所望の活性を有する抗ヒトC R T H 2抗体、該抗体断片、該抗体のアミノ酸配列をコードするDNA、該DNAを含むベクター、該抗体を生産するハイブリドーマおよび抗体生産細胞、該抗体の製造方法、該抗体または抗体断片を含む組成物、該抗体または抗体断片を用いるアレルギー性疾患、自己免疫疾患、好酸球増多や機能亢進を伴う疾患、T h 2細胞の増多や機能亢進を伴う疾患などの治療方法および診断方法、並びに該抗体または抗体断片を含む医薬および診断薬を提供することである。

課題を解決するための手段

[0017] 本発明は、以下の(1)～(26)に関する。

(1) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の192番目のグリシンおよび194番目のアスパラギン酸の少なくとも一方を認識し、結合する抗体または該抗体断片。

(2) 抗体が、配列番号2で表されるヒトC R T H 2のアミノ酸配列の12番目のプロリン、13番目のイソロイシン、14番目のロイシン、15番目のグルタミン酸、177番目のアスパラギン酸、178番目のグリシン、179番目のアルギニン、180番目のイソロイシン、181番目のメチオニ

ン、１８２番目のシステイン、１８３番目のチロシン、１８４番目のチロシン、１８５番目のアスパラギン、１８６番目のバリン、１８７番目のロイシン、１８８番目のロイシン、１８９番目のロイシン、１９５番目のアルギニン、１９６番目のアスパラギン酸、１９７番目のアラニン、および１９８番目のスレオニンからなる群から選ばれるアミノ酸残基の少なくとも１つを認識し、結合する抗体である（１）に記載の抗体または該抗体断片。

（３）抗体が、以下の（ａ）～（ｇ）からなる群から選ばれるアミノ酸残基の少なくとも１つを認識する抗体である、（１）または（２）に記載の抗体または該抗体断片。

（ａ）配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１２番目のプロリン、１４番目のロイシンおよび１５番目のグルタミン酸、

（ｂ）配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１７７番目のアスパラギン酸、１７８番目のグリシン、および１７９番目のアルギニン、

（ｃ）配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１８０番目のイソロイシンおよび１８１番目のメチオニン、

（ｄ）配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１８３番目のチロシン、１８４番目のチロシンおよび１８５番目のアスパラギン、

（ｅ）配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１８７番目のロイシン、１８８番目のロイシンおよび１８９番目のロイシン、

（ｆ）配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１９５番目のアルギニン、並びに

（ｇ）配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１９６番目のアスパラギン酸および１９８番目のスレオニン。

（４）抗体が、以下の（ａ）～（ｄ）からなる群から選ばれるいずれか１つの抗体である、（１）～（３）のいずれか１に記載の抗体または該抗体断片。

（ａ）抗体重鎖可変領域（以下、ＶＨと略記する）の相補性決定領域（以下、ＣＤＲと略記する）１～３が、それぞれ配列番号２０～２２で表されるア

ミノ酸配列を含み、かつ抗体軽鎖可変領域（以下、V Lと略記する）のC D R 1～3が、それぞれ配列番号23～25で表されるアミノ酸配列を含む抗体、

（b）配列番号49で表されるアミノ酸配列または配列番号49で表されるアミノ酸配列中の18番目のロイシンをメチオニンに、77番目のアスパラギンをセリンに、93番目のバリンをスレオニンに、および117番目のスレオニンをバリンに置換する改変から選ばれる少なくとも1つの改変が導入されたアミノ酸配列を含むV H並びに配列番号33で表されるアミノ酸配列または配列番号33で表されるアミノ酸配列中の2番目のイソロイシンをバリンに、4番目のメチオニンをロイシンに、15番目のプロリンをロイシンに、および85番目のアラニンをプロリンに置換する改変から選ばれる少なくとも1つの改変が導入されたアミノ酸配列を含むV Lを含む抗体、

（c）配列番号49、51、53、55、57および59で表されるアミノ酸配列のいずれか1つを含むV H並びに配列番号33、35、37、39、41、43、45および47で表されるアミノ酸配列のいずれか1つを含むV Lを含む抗体、並びに

（d）配列番号17で表されるアミノ酸配列を含むV Hおよび配列番号19で表されるアミノ酸配列を含むV Lを含む抗体。

（5）抗体が、下記（a）～（h）からなる群から選ばれる少なくとも1つの特徴を有する抗体である（1）～（4）のいずれか1に記載の抗体または該抗体断片。

（a）ヒトC R T H 2のリガンド存在下でヒトC R T H 2に対する反応性が低下しない、

（b）中和活性を有しない、

（c）抗体依存性細胞傷害（A D C C）活性を有する、

（d）マスト細胞およびT h 1細胞の少なくとも一方に反応しない、

（e）好酸球、好塩基球、T h 2細胞および2型自然リンパ球（I L C 2）から選ばれる少なくとも一つの細胞に反応する。

- (f) アゴニスト活性を有しない、
- (g) ヒトC R T H 2のリガンドによるシグナルを増強しない、並びに
- (h) 活性化状態または不活性化状態のヒトC R T H 2に対する反応性が変化しない。
- (6) 抗体が、ヒトF c領域を含む抗体である、(1)～(5)のうちのいずれか1に記載の抗体または該抗体断片。
- (7) 抗体が、モノクローナル抗体である(1)～(6)のいずれか1に記載の抗体または該抗体断片。
- (8) 抗体が、遺伝子組換え抗体である(1)～(7)のいずれか1に記載の抗体または該抗体断片。
- (9) 遺伝子組換え抗体が、ヒト型キメラ抗体、ヒト型C D R移植抗体およびヒト抗体から選ばれるいずれか1つの遺伝子組換え抗体である、(8)に記載の遺伝子組換え抗体または該抗体断片。
- (10) 抗体が、サルC R T H 2に結合する抗体である(1)～(9)のいずれか1つに記載の抗体または該断片。
- (11) F a b、F a b'、F (a b')₂、s c F v、d i a b o d y、d s F vおよびC D Rを含むペプチドから選ばれるいずれか1つの抗体断片である(1)～(10)のいずれか1に記載の該抗体断片。
- (12) (1)～(11)のいずれか1に記載の抗体または該抗体断片を産生するハイブリドーマ。
- (13) (1)～(11)のいずれか1に記載の抗体または該抗体断片をコードするD N A。
- (14) (13)に記載のD N Aを含有する組換え体ベクター。
- (15) (14)に記載の組換え体ベクターを宿主細胞に導入して得られる形質転換株。
- (16) (12)に記載のハイブリドーマまたは(15)に記載の形質転換株を培地に培養し、培養物中に(1)～(11)のいずれか1に記載の抗体または該抗体断片を生産蓄積させ、該培養物から抗体または該抗体断片を採

取することを特徴とする（１）～（１１）のいずれか１に記載の抗体または該抗体断片の製造方法。

（１７）（１）～（１１）のいずれか１に記載の抗体または該抗体断片を有効成分として含有する、ヒトＣＲＴＨ２が関係する疾患の治療剤。

（１８）（１）～（１１）のいずれか１に記載の抗体または該抗体断片を有効成分として含有する、ヒトＣＲＴＨ２が関係する疾患の診断剤。

（１９）ＣＲＴＨ２が関係する疾患がアレルギー性疾患、自己免疫疾患、好酸球増多および機能亢進の少なくとも一方を伴う疾患、Ｔｈ２細胞の増多および機能亢進の少なくとも一方を伴う疾患、または２型自然リンパ球（ＩＬＣ２）の増多および機能亢進の少なくとも一方を伴う疾患である（１７）または（１８）に記載の剤。

（２０）（１）～（１１）のいずれか１に記載の抗体または該抗体断片の有効量を投与することを含む、ヒトＣＲＴＨ２が関係する疾患の治療方法。

（２１）（１）～（１１）のいずれか１に記載の抗体または該抗体断片の有効量を投与することを含む、ヒトＣＲＴＨ２が関係する疾患の診断方法。

（２２）ヒトＣＲＴＨ２が関係する疾患がアレルギー性疾患、自己免疫疾患、好酸球増多および機能亢進の少なくとも一方を伴う疾患、Ｔｈ２細胞の増多および機能亢進の少なくとも一方を伴う疾患、またはＩＬＣ２の増多および機能亢進の少なくとも一方を伴う疾患である（２０）または（２１）に記載の方法。

（２３）ヒトＣＲＴＨ２が関係する疾患の治療および診断の少なくとも一方に使用するための、（１）～（１１）のいずれか１に記載の抗体または該抗体断片。

（２４）ヒトＣＲＴＨ２が関係する疾患がアレルギー性疾患、自己免疫疾患、好酸球増多および機能亢進の少なくとも一方を伴う疾患、Ｔｈ２細胞の増多および機能亢進の少なくとも一方を伴う疾患、またはＩＬＣ２の増多および機能亢進の少なくとも一方を伴う疾患である（２３）に記載の抗体または該抗体断片。

(25) ヒトC R T H 2 が関係する疾患の治療および診断剤の少なくとも一方の製造のための、(1) ~ (11) のいずれか1に記載の抗体または該抗体断片の使用。

(26) ヒトC R T H 2 が関係する疾患がアレルギー性疾患、自己免疫疾患、好酸球増多および機能亢進の少なくとも一方を伴う疾患、T h 2 細胞の増多および機能亢進の少なくとも一方を伴う疾患、またはI L C 2 の増多および機能亢進の少なくとも一方を伴う疾患である(25)に記載の使用。

発明の効果

[0018] 本発明により配列番号2で表されるヒトC R T H 2 のアミノ酸配列の192番目のグリシンおよび194番目のアスパラギン酸の少なくとも一方を認識し、結合する抗体または該抗体断片などが提供される。

[0019] 本発明の抗体は、好酸球、好塩基球T h 2 細胞、I L C 2 などのC R T H 2 を発現する細胞に特異的に反応し、高濃度のリガンド存在下においてもC R T H 2 発現細胞に高い反応性を示すとともに、アゴニスト活性、中和活性、およびヒトC R T H 2 のリガンドによるシグナルの増強活性を有しない。したがって本発明の抗体または該抗体断片は、C R T H 2 が発現する好酸球、好塩基球、T h 2 細胞、I L C 2 などのC R T H 2 を発現する細胞を標的とする治療効果を発揮し得る。

図面の簡単な説明

[0020] [図1]図1は、シグナル配列を含まないL y m 2 抗体軽鎖可変領域及び各ヒト化L y m 2 抗体軽鎖可変領域(L V 0、L V 1、L V 2 a、L V 2 b、L V 2 c、L V 3 a、L V 3 bおよびL V 4)のアミノ酸配列を示す。各配列中の枠で囲まれた領域は、C D R 配列を示す。

[図2]図2は、シグナル配列を含まないL y m 2 抗体重鎖可変領域及び各ヒト化L y m 2 抗体重鎖可変領域(H V 0、H V 1、H V 2 a、H V 2 b、H V 3 およびH V 4)のアミノ酸配列を示す。各配列中の枠で囲まれた領域は、C D R 配列を示す。

[図3]図3 (A) ~ (C) は、ラット/ヒトキメラ型L y m 2 抗体(以下、c

h L y m 2 と記す場合もある) およびヒト化 L y m 2 抗体のヒト好酸球およびヒト好塩基球に対する細胞傷害活性をフローサイトメトリーで解析した結果を示す。図 3 (A) ~ (C) において、それぞれ、左側の図はヒト好酸球に対する細胞傷害活性を、右側の図はヒト好塩基球に対する細胞傷害活性を示す。それぞれの図において縦軸はコントロールビーズ 2000 個あたりの各細胞の個数を、横軸は抗体濃度を示す。図 3 (A) ○は c h L y m 2、●はヒト化 L y m 2 抗体 L V O H V 0、△はアイソタイプコントロール抗体を示す。図 3 (B) ○は c h L y m 2、■はヒト化 L y m 2 抗体 L V O H V 1、△はアイソタイプコントロール抗体を示す。図 3 (C) ○は c h L y m 2、▲はヒト化 L y m 2 抗体 L V O H V 2 a、△はアイソタイプコントロール抗体を示す。

[図4]図 4 (A) はヒト化 L y m 2 抗体 L V O H V 1、図 4 (B) は c h L y m 2 の各ヒト C R T H 2 アミノ酸置換体発現細胞に対する反応性をそれぞれ示したものである。それぞれの図において、縦軸はアザミグリーンタグの蛍光強度で、各抗ヒト C R T H 2 モノクローナル抗体の蛍光強度を補正した相対的蛍光強度に関して、野生型ヒト C R T H 2 発現細胞に対する反応性の値を 100% とした際の、各アミノ酸置換体発現細胞に対する反応性の値 (%) を示す。横軸において W T は野生型ヒト C R T H 2 を示し、それ以外は、アミノ酸置換体の種類を示す。* は野生型 C R T H 2 の相対的蛍光強度から 90% 以上の相対的蛍光強度の低下が認められたことを意味する。以下、図 5 ~ 図 7 も同様である。

[図5]図 5 (A) は h u 19 A 2 v 5 2、図 5 (B) は h u 8 B 1 v 1 の各 C R T H 2 アミノ酸置換体に対する反応性をそれぞれ示したものである。

[図6]図 6 (A) は c h 3 C 1 2、図 6 (B) は c h 3 1 A 5 の各 C R T H 2 アミノ酸置換体に対する反応性をそれぞれ示したものである。

[図7]図 7 は、B M 1 6 の各 C R T H 2 アミノ酸置換体に対する反応性を示したものである。

[図8]図 8 は、ヒト好酸球に対する抗ヒト C R T H 2 モノクローナル抗体の反

応性をフローサイトメトリーで解析した結果を示す。●はL y m 2 抗体、○はB M 1 6、▲は3 0 1 1 0 8を示し、縦軸は蛍光強度を、横軸は各抗ヒトC R T H 2モノクローナル抗体の抗体濃度を示す。

[図9]図9は、ヒト好塩基球に対するc h L y m 2の反応性をフローサイトメトリーで解析した結果を示す。塗りつぶし部分がアイソタイプコントロール抗体の反応性、実線で囲まれた部分がc h L y m 2の反応性を、それぞれ示し、縦軸は細胞数を、横軸は蛍光強度を示す。

[図10]図10は、ヒトC D 4陽性T細胞に対するヒト化L y m 2抗体L V O H V 1の反応性をフローサイトメトリーで解析した結果を示す。F o r w a r d s c a t t e r（以下、F S Cと記す）－S i d e s c a t t e r（以下、S S Cと記す）展開によりリンパ球を分画し、さらにC D 3陽性かつC D 4陽性細胞で分画した細胞群に対する、ヒト化L y m 2抗体L V O H V 1による蛍光染色の蛍光強度およびC D 4抗体による蛍光染色の蛍光強度をそれぞれ縦軸および横軸に示す。

[図11]図11（A）および図11（B）は、抗ヒトC R T H 2モノクローナル抗体のヒト好酸球およびヒト好塩基球に対する細胞傷害活性をフローサイトメトリーで解析した結果を示す。上段はヒト好酸球、下段はヒト好塩基球に対する細胞傷害活性を示す。それぞれの図において、縦軸にコントロールビーズ1000個あたりの各細胞の個数を、横軸に抗体濃度を示す。○はヒト化L y m 2抗体L V O H V 1、□はh u 1 9 A 2 v 5 2、▲はh u 8 B 1 v 1、△はc h 3 C 1 2、◆はc h 3 1 A 5、×はアイソタイプコントロール抗体を示す。

[図12]図12（A）および図12（B）は、抗ヒトC R T H 2モノクローナル抗体のヒトT h 2およびT h 1サイトカイン減少活性を解析した結果を示す。図12（A）は各抗体を添加した時のT h 2サイトカインであるI L - 5またはI L - 1 3の濃度を縦軸に示す。また図12（B）は各抗体を添加した時の、T h 1サイトカインであるI F N - γ の濃度を縦軸に示す。

[図13]図13（A）～図13（C）は、C R T H 2リガンドであるD K P G

D 2 存在下における抗ヒトC R T H 2 モノクローナル抗体の反応性変化を、ヒトC R T H 2 発現2 9 3 E B N A 細胞を用いて、フローサイトメトリーで解析した結果を示す。凡例に示す抗ヒトC R T H 2 モノクローナル抗体の濃度が0. 3 μ g / mL、1 μ g / mL、および3 μ g / mLにおける結果をそれぞれ図1 3 (A)、図1 3 (B) および図1 3 (C) に示す。それぞれの図において、縦軸はD K P G D 2 非存在下での蛍光強度を1 0 0 %としたときの蛍光強度の割合を示す。

[図14]図1 4 は、I g E およびクロスリンク抗体処理により刺激したヒト分化誘導マスト細胞に対する抗ヒトC R T H 2 モノクローナル抗体の反応性をフローサイトメトリーで解析した結果を示す。それぞれの図は、図の上を示す抗体の反応性を示しており、縦軸に細胞数を、横軸に蛍光強度を示す。塗りつぶし部分がアイソタイプコントロール抗体の反応性を、実線で囲まれた部分が抗ヒトC R T H 2 モノクローナル抗体の反応性を、それぞれ示す。

[図15]図1 5 は、ヒト分化誘導T h 1 細胞に対する抗ヒトC R T H 2 モノクローナル抗体の反応性をフローサイトメトリーで解析した結果を示す。それぞれの図は、図の上を示す抗体の反応性を示しており、縦軸に細胞数を、横軸に蛍光強度を示す。塗りつぶし部分がアイソタイプコントロール抗体の反応性を、実線で囲まれた部分が抗ヒトC R T H 2 モノクローナル抗体の反応性を、それぞれ示す。

[図16]図1 6 は、ヒト好酸球の形態変化を指標にしたL y m 2 抗体のアntagオニスト活性評価の結果を示す。グラフの下に示す各抗体の存在下または非存在下で、凡例に示す濃度のD K P G D 2 を処理した際に、フローサイトメーター解析における高F S C 領域に検出される好酸球の割合(%)を縦軸に示す。

[図17]図1 7 は、ヒト好酸球の形態変化を指標にしたL y m 2 抗体のアntagオニスト活性評価の結果を示す。凡例に示す濃度のL y m 2 抗体を処理した際に、高F S C 領域に検出される好酸球の割合(%)を縦軸に示す。

[図18]図1 8 (A) ~ 図1 8 (C) はいずれも、ヒト好酸球の形態変化を指

標にした抗ヒトC R T H 2モノクローナル抗体のアゴニスト活性、アンタゴニスト活性およびリガンドによる活性化の増強活性の評価の結果を示す。図18 (A) はヒト化抗体またはキメラ抗体、図18 (B) はラット抗体、図18 (C) はマウス抗体についての結果をそれぞれ示す。各図において、縦軸は、D K P G D 2の存在、または非存在下で、凡例に示す各抗ヒトC R T H 2モノクローナル抗体またはアイソタイプ抗体を処理した際に、フローサイトメーター解析における高F S C領域に検出される好酸球の割合(%)を示す。

[図19]図19はC R T H 2発現細胞の膜画分へのG T P γ SまたはG D P処理による、C R T H 2のコンフォメーションの変化がC R T H 2モノクローナル抗体の反応性に与える影響を、E L I S A法により解析した結果を示す。縦軸はG T P γ SおよびG D P未処理時の吸光度を1とした際のF o l d c h a n g eを示す。横軸はG T P γ SおよびG D P処理の有無、および、評価抗体(h u 1 9 A 2 v 5 2およびヒト化L y m 2抗体L V O H V 1)を示す。

[図20]図20は、アザミグリーン融合ヒトC R T H 2発現C H O / D G 4 4細胞およびカニクイザルC R T H 2発現C H O / D G 4 4細胞におけるアザミグリーンの発現を、フローサイトメトリーで解析した結果を示す。縦軸に細胞数を、横軸にアザミグリーンの蛍光強度を示す。塗りつぶし部分が親細胞であるC H O / D G 4 4細胞における蛍光強度を、実線で囲まれた部分がアザミグリーン融合ヒトC R T H 2発現C H O / D G 4 4細胞における蛍光強度を、点線で囲まれた部分がアザミグリーン融合カニクイザルC R T H 2発現C H O / D G 4 4細胞における蛍光強度をそれぞれ示す。

[図21]アザミグリーン融合ヒトC R T H 2発現C H O / D G 4 4細胞およびカニクイザルC R T H 2発現C H O / D G 4 4細胞に対するヒト化L y m 2抗体L V O H V 1およびアイソタイプ抗体の反応性をフローサイトメトリーで解析した結果を示す。○はアザミグリーン融合ヒトC R T H 2発現C H O / D G 4 4細胞に対するL V O H V 1の反応性、●はアザミグリーン融合カニクイザルC R T H 2発現C H O / D G 4 4細胞に対するL V O H V 1の反応性を示す。

ニクイザルC R T H 2 発現C H O / D G 4 4 細胞に対するL V O H V 1 の反応性、△はアザミグリーン融合ヒトC R T H 2 発現C H O / D G 4 4 細胞に対するアイソタイプ抗体の反応性、▲はアザミグリーン融合カニクイザルC R T H 2 発現C H O / D G 4 4 細胞に対するアイソタイプ抗体の反応性を示し、縦軸は蛍光強度を、横軸は各抗体の抗体濃度を示す。

発明を実施するための形態

- [0021] 本発明におけるヒトC R T H 2 としては、配列番号2またはG e n B a n k アクセッション番号B A A 7 4 5 1 8 で表されるアミノ酸配列を含むポリペプチドが挙げられる。配列番号2またはG e n B a n k アクセッション番号B A A 7 4 5 1 8 で表されるアミノ酸配列において、1 以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列からなり、かつヒトC R T H 2 の機能を有するポリペプチド並びに配列番号2またはG e n B a n k アクセッション番号B A A 7 4 5 1 8 で表されるアミノ酸配列と60%以上、好ましくは80%以上、より好ましくは90%以上、さらに好ましくは95%以上、最も好ましくは98%以上の相同性を有するアミノ酸配列からなり、かつヒトC R T H 2 の機能を有するポリペプチドも、本発明におけるヒトC R T H 2 に包含される。
- [0022] 配列番号2またはG e n B a n k アクセッション番号B A A 7 4 5 1 8 で表されるアミノ酸配列において、1 以上のアミノ酸が欠失、置換、挿入および／または付加されたアミノ酸配列を有するポリペプチドは、部位特異的変異導入法 [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)、Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997)、Nucleic Acids Research, 10, 6487 (1982)、Proc. Natl. Acad. Sci. USA, 79, 6409 (1982)、Gene, 34, 315 (1985)、Nucleic Acids Research, 13, 4431 (1985)、Proc. Natl. Acad. Sci. USA, 82, 488 (1985)] などを用いて、例えば配列番号2で表されるアミノ酸配列を有するポリペプチドをコードするD N A に部位特異的変異を導入することにより得ることができる。欠失、置換または付加されるアミ

ノ酸の数は特に限定されないが、好ましくは1個～数十個、例えば、1～20個、より好ましくは1個～数個、例えば、1～5個のアミノ酸である。

[0023] ヒトC R T H 2をコードする遺伝子としては、G e n B a n k アクセション番号A B O O 8 5 3 5または配列番号1で表される塩基配列が挙げられる。G e n B a n k アクセション番号A B O O 8 5 3 5または配列番号1で表される塩基配列において、1以上の塩基が欠失、置換または付加された塩基配列からなり、かつヒトC R T H 2の機能を有する蛋白質をコードするD N Aを含む遺伝子、G e n B a n k アクセション番号A B O O 8 5 3 5または配列番号1で表される塩基配列と少なくとも60%以上の相同性を有する塩基配列、好ましくは80%以上の相同性を有する塩基配列、さらに好ましくは95%以上の相同性を有する塩基配列からなり、かつヒトC R T H 2の機能を有するポリペプチドをコードするD N Aを含む遺伝子、並びに配列番号1で表される塩基配列を有するD N Aとストリンジェントな条件下でハイブリダイズするD N Aからなり、かつヒトC R T H 2の機能を有するポリペプチドをコードするD N Aを含む遺伝子なども本発明のC R T H 2をコードする遺伝子に包含される。

[0024] ストリンジェントな条件下でハイブリダイズするD N Aとしては、配列番号1で表される塩基配列を有するD N Aをプローブに用いた、コロニー・ハイブリダイゼーション法、プラーク・ハイブリダイゼーション法、サザンブロット・ハイブリダイゼーション法、またはD N Aマイクロアレイ法などにより得られるハイブリダイズ可能なD N Aを意味する。

[0025] 具体的には、ハイブリダイズしたコロニー若しくはプラーク由来のD N A、または該配列を有するP C R産物もしくはオリゴD N Aを固定化したフィルターまたはスライドガラスを用いて、0.7～1.0 m o l / L の塩化ナトリウム存在下、65℃でハイブリダイゼーション [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)、Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997)、DNA Cloning 1: Core Techniques, A Practical Approach, Se

cond Edition, Oxford University, (1995)] を行った後、0.1～2倍濃度のSSC溶液（1倍濃度のSSC溶液の組成は、150mmol/L塩化ナトリウム、15mmol/Lクエン酸ナトリウムよりなる）を用い、65℃条件下でフィルターまたはスライドガラスを洗浄することにより同定できるDNAを挙げることができる。

[0026] ハイブリダイズ可能なDNAとしては、GenBankアクセション番号AB008535または配列番号1で表される塩基配列と少なくとも60%以上の相同性を有するDNA、好ましくは80%以上の相同性を有するDNA、さらに好ましくは95%以上の相同性を有するDNAを挙げることができる。

[0027] 真核生物の蛋白質をコードする遺伝子の塩基配列には、しばしば遺伝子の多型が認められる。本発明において用いられる遺伝子に、このような多型によって塩基配列に小規模な変異を生じた遺伝子も、本発明のヒトC R T H 2をコードする遺伝子に包含される。

[0028] 本発明における相同性の数値は、特に明示した場合を除き、当業者に公知の相同性検索プログラムを用いて算出される数値であってよいが、塩基配列については、BLAST [J. Mol. Biol., 215, 403 (1990)] においてデフォルトのパラメータを用いて算出される数値など、アミノ酸配列については、BLAST2 [Nucleic Acids Res., 25, 3389 (1997)、Genome Res., 7, 649 (1997)、<http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/information3.html>] においてデフォルトのパラメータを用いて算出される数値などが挙げられる。

[0029] デフォルトのパラメータとしては、G (Cost to open gap) が塩基配列の場合は5、アミノ酸配列の場合は11、-E (Cost to extend gap) が塩基配列の場合は2、アミノ酸配列の場合は1、-q (Penalty for nucleotide mismatch) が-3、-r (reward for nucleotide match) が1、-e (expect value) が10、-W (wor

d s i z e) が塩基配列の場合は 11 残基、アミノ酸配列の場合は 3 残基、
 -y [D r o p o f f (X) f o r b l a s t e x t e n s i o n s
 i n b i t s] が b l a s t n の場合は 20、b l a s t n 以外のプロ
 グラムでは 7、-X (X d r o p o f f v a l u e f o r g a p p
 e d a l i g n m e n t i n b i t s) が 15 および -Z (f i n a
 l X d r o p o f f v a l u e f o r g a p p e d a l i g n
 m e n t i n b i t s) が b l a s t n の場合は 50、b l a s t n 以
 外のプログラムでは 25 である ([http://www.ncbi.nlm.nih.gov/blast/html/
 blastcgihelp.html](http://www.ncbi.nlm.nih.gov/blast/html/blastcgihelp.html))。

[0030] 配列番号 2 または G e n B a n k アクセッション番号 B A A 7 4 5 1 8 で
 示されるアミノ酸配列の部分配列からなるポリペプチドは、当業者に公知の
 方法によって作製することができる。例えば、配列番号 2 で表されるアミノ
 酸配列をコードする DNA の一部を欠失させ、これを含む発現ベクターを導入
 した形質転換体を培養することにより作製することができる。また、上記
 の方法で作製されるポリペプチドまたは DNA に基づいて、上記と同様の方
 法により、配列番号 2 または G e n B a n k アクセッション番号 B A A 7 4
 5 1 8 で示されるアミノ酸配列の部分配列において 1 以上のアミノ酸が欠失
 、置換または付加されたアミノ酸配列を有するポリペプチドを得ることがで
 きる。さらに、配列番号 2 または G e n B a n k アクセッション番号 B A A
 7 4 5 1 8 で示されるアミノ酸配列の部分配列からなるポリペプチド、また
 は配列番号 2 または G e n B a n k アクセッション番号 B A A 7 4 5 1 8 で
 示されるアミノ酸配列の部分配列において 1 以上のアミノ酸が欠失、置換ま
 たは付加されたアミノ酸配列を有するポリペプチドは、フルオレニルメチル
 オキシカルボニル (F m o c) 法、t -ブチルオキシカルボニル (t B o c
) 法などの化学合成法によって製造することもできる。

[0031] ヒト C R T H 2 としての機能としては、そのリガンド、例えば P G D 2 と
 の結合により、ヒト C R T H 2 依存的な細胞内シグナルが伝達され、ヒト C
 R T H 2 を発現する細胞の遊走、該細胞からのサイトカイン産生亢進、また

は細胞径、細胞表面積などの変化を伴う細胞形態変化が誘導されることなどが挙げられる。

[0032] ヒトC R T H 2の細胞外領域としては、配列番号2で表されるヒトC R T H 2のアミノ酸配列の1～33番目のアミノ酸残基を含むN末領域、95～111番目のアミノ酸残基を含むループ1領域、169～206番目のアミノ酸残基を含むループ2領域および264～285番目のアミノ酸残基を含むループ3領域が挙げられる [J Immunol, 1999, 162(3): p.1278-86.]。N末領域、ループ1領域、ループ2領域およびループ3領域として、具体的には、それぞれ、配列番号2で表されるアミノ酸配列における1～33番目、95～111番目、169～206番目および264～285番目のアミノ酸残基を含むポリペプチド部分が挙げられる。

[0033] 本発明における抗体としては、モノクローナル抗体、ポリクローナル抗体等いずれの抗体であってもよいが、好ましくはモノクローナル抗体が挙げられる。本発明の抗体として具体的には、ハイブリドーマにより産生される抗体、または遺伝子組換え技術によって産生される遺伝子組換え抗体を挙げることができる。また遺伝子組換え抗体としては、例えば、遺伝子組換え技術によって作製されるマウス抗体、ラット抗体、ヒト型キメラ抗体、ヒト化抗体及びヒト抗体などが挙げられる。

[0034] モノクローナル抗体とは、単クローンの抗体産生細胞が分泌する抗体であり、ただ一つのエピトープ（抗原決定基ともいう）を認識し、モノクローナル抗体を構成するアミノ酸配列（1次構造）が均一である。

[0035] 本発明においてモノクローナル抗体としては、ハイブリドーマにより生産される抗体、または抗体遺伝子を含む発現ベクターで形質転換した形質転換体により生産される抗体など、遺伝子組換え技術によって作製される遺伝子組換え抗体を挙げることができる。

[0036] ポリクローナル抗体とは、2つ以上のモノクローナル抗体が含まれる抗体群であり、その抗体群を構成する複数の抗体によって複数のエピトープを認識することができる。

本発明においてエピトープとしては、モノクローナル抗体が認識し、結合する単一のアミノ酸配列およびアミノ酸配列からなる立体構造並びに翻訳後修飾により修飾されたアミノ酸配列および該アミノ酸配列からなる立体構造などが挙げられる。

[0037] 翻訳後修飾により修飾されたアミノ酸配列としては、糖鎖がOH置換基を有するスレオニンおよびセリンに結合したO結合型糖鎖、NH₂置換基を有するグルタミンおよびアスパラギンに結合したN結合型糖鎖並びに硫酸分子がOH置換基を有するスレオニンに結合した硫酸基などが結合したアミノ酸配列が挙げられる。

[0038] 本発明の抗体が認識するヒトCRTH2のエピトープは、ヒトCRTH2の一部のドメインを欠失させた欠損体、ヒトCRTH2の一部のアミノ酸残基を他のアミノ酸残基に置換させた変異体、他のタンパク質由来のドメインと置換させた変異体およびヒトCRTH2の部分ペプチド断片等を用いた抗体の結合実験を行うことにより決定することができる。また、本発明の抗体が結合するヒトCRTH2のエピトープは、タンパク質分解酵素にて消化したヒトCRTH2に本発明の抗体を添加し、既知の質量分析法を用いたエピトープマッピングを行うことにより決定することができる。

[0039] 本発明の抗体が認識するヒトCRTH2のエピトープに含まれるアミノ酸残基としては、例えば該アミノ酸残基の置換により、本発明の抗体の反応性が消失するアミノ酸残基が挙げられる。

[0040] 本発明における抗体の反応性は、例えば、野生型ヒトCRTH2受容体またはアミノ酸置換体を発現する細胞に対する抗体の結合量（野生型及び置換体の発現量に応じて補正される）をフローサイトメトリー等を用いて測定することによって求めることができる。また、抗体の結合量は、固相サンドイッチ法などを用いたラジオイムノアッセイ、または酵素免疫測定法（ELISA）などを用いたヒトCRTH2に対する公知の免疫学的検出法、またはBiacoreシステム（ジーイーヘルスケア社）などを用いた表面プラズモン共鳴などの方法で確認することができる。

[0041] また、公知の免疫学的検出法 [Monoclonal Antibodies-Principles and Practice, Third edition, Academic Press(1996)、Antibodies—A Laboratory Manual, Cold Spring Harbor Laboratory(1988)、単クローン抗体実験マニュアル, 講談社サイエンティフィック(1987)] などを組み合わせて確認することもできる。

[0042] 本発明における抗体の反応性の消失とは、野生型ヒトC R T H 2を発現する細胞に対する抗体の反応性と比較して、アミノ酸置換体を発現する細胞に対する抗体の反応性が70%以上、好ましくは80%以上、より好ましくは90%以上、さらに好ましくは95%以上低下することを示す。

[0043] 本発明の抗体が結合するエピトープとしては、配列番号2で表されるヒトC R T H 2のアミノ酸配列の192番目のグリシンおよび194番目のアスパラギン酸の少なくとも1つのアミノ酸残基を含むエピトープが挙げられる。

[0044] また、本発明の抗体が結合するエピトープとして、具体的には、下記の（a）～（c）のエピトープが挙げられる。

（a）配列番号2で表されるヒトC R T H 2のアミノ酸配列の192番目のグリシンを含むエピトープ、

（b）配列番号2で表されるヒトC R T H 2のアミノ酸配列の194番目のアスパラギン酸を含むエピトープ、

（c）配列番号2で表されるヒトC R T H 2のアミノ酸配列の192番目のグリシンおよび194番目のアスパラギン酸を含むエピトープ。

[0045] また、本発明の抗体が結合するエピトープとしては、配列番号2で表されるヒトC R T H 2のアミノ酸配列の192番目のグリシンおよび194番目のアスパラギン酸の少なくとも1つのアミノ酸残基を含み、かつ配列番号2で表されるヒトC R T H 2のアミノ酸配列の12番目のプロリン、14番目のロイシン、15番目のグルタミン酸、177番目のアスパラギン酸、178番目のグリシン、179番目のアルギニン、180番目のイソロイシン、181番目のメチオニン、183番目のチロシン、184番目のチロシン、

185番目のアスパラギン、187番目のロイシン、188番目のロイシン、189番目のロイシン、195番目のアルギニン、196番目のアスパラギン酸、および198番目のスレオニンからなる群から選ばれるアミノ酸残基の少なくとも1つを含むエピトープが挙げられる。

[0046] また、本発明の抗体が結合するエピトープとしては配列番号2で表されるヒトC R T H 2のアミノ酸配列の192番目のグリシンおよび194番目のアスパラギン酸の少なくとも1つのアミノ酸残基を含み、かつ下記の(a)～(g)の少なくともいずれか1つを含むエピトープが挙げられる。

(a) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の12番目のプロリン、14番目のロイシンおよび15番目のグルタミン酸、

(b) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の177番目のアスパラギン酸、178番目のグリシン、および179番目のアルギニン、

(c) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の180番目のイソロイシンおよび181番目のメチオニン、

(d) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の183番目のチロシン、184番目のチロシンおよび185番目のアスパラギン、

(e) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の187番目のロイシン、188番目のロイシンおよび189番目のロイシン、

(f) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の195番目のアルギニン、並びに

(g) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の196番目のアスパラギン酸および198番目のスレオニン。

[0047] また、本発明の抗体が結合するエピトープに含まれる他のアミノ酸残基としては、本発明の抗体がC R T H 2へ結合する際に、配列番号2で表されるヒトC R T H 2のアミノ酸配列に存在し、かつ実質的に認識し結合しているアミノ酸残基であればいずれのアミノ酸残基でもよく、具体的には配列番号2で表されるヒトC R T H 2のアミノ酸配列の12番目のプロリン、14番目のロイシン、15番目のグルタミン酸、177番目のアスパラギン酸、1

78番目のグリシン、179番目のアルギニン、180番目のイソロイシン、181番目のメチオニン、183番目のチロシン、184番目のチロシン、185番目のアスパラギン、187番目のロイシン、188番目のロイシン、189番目のロイシン、192番目のグリシン、194番目のアスパラギン酸、195番目のアルギニン、196番目のアスパラギン酸、および198番目のスレオニンからなる群から選ばれるアミノ酸残基と立体構造上近接して存在しているアミノ酸残基、並びに配列番号2で表されるヒトC R T H 2のアミノ酸配列の12番目のプロリン、14番目のロイシン、15番目のグルタミン酸、177番目のアスパラギン酸、178番目のグリシン、179番目のアルギニン、180番目のイソロイシン、181番目のメチオニン、183番目のチロシン、184番目のチロシン、185番目のアスパラギン、187番目のロイシン、188番目のロイシン、189番目のロイシン、192番目のグリシン、194番目のアスパラギン酸、195番目のアルギニン、196番目のアスパラギン酸、および198番目のスレオニンからなる群から選ばれるアミノ酸から選ばれるアミノ酸残基と1次配列上近接しているアミノ酸残基などが挙げられる。

[0048] 抗体分子はイムノグロブリン（以下、I gと表記する）とも称され、ヒト抗体は、分子構造の違いに応じて、I g A 1、I g A 2、I g D、I g E、I g G 1、I g G 2、I g G 3、I g G 4およびI g Mのアイソタイプに分類される。アミノ酸配列の相同性が比較的高いI g G 1、I g G 2、I g G 3およびI g G 4を総称してI g Gともいう。

[0049] 抗体分子は重鎖（Heavy chain、以下H鎖と記す）および軽鎖（Light chain、以下L鎖と記す）と呼ばれるポリペプチドより構成される。また、H鎖はN末端側よりH鎖可変領域（V Hとも表記される）、H鎖定常領域（C Hとも表記される）、L鎖はN末端側よりL鎖可変領域（V Lとも表記される）、L鎖定常領域（C Lとも表記される）の各領域により、それぞれ構成される。

[0050] C Hは各サブクラスごとに、 α 、 δ 、 ϵ 、 γ および μ 鎖がそれぞれ知られ

ている。C Hはさらに、N末端側よりC H 1ドメイン、ヒンジドメイン、C H 2ドメイン、C H 3ドメインの各ドメインにより構成される。ドメインとは、抗体分子の各ポリペプチドを構成する機能的な構造単位をいう。また、C H 2ドメインとC H 3ドメインを併せてF c領域または単にF cという。C Lは、C λ鎖およびC κ鎖が知られている。

[0051] 本発明の抗体におけるC Hとしては、I gに属すればいかなるものでもよいが、I g Gクラスのもの好適であり、さらにI g Gクラスに属するI g G 1、I g G 2、I g G 3、I g G 4といったサブクラスのいずれも用いることができる。

[0052] 本発明の抗体におけるC Lのアミノ酸配列としては、ヒト抗体のアミノ酸配列または非ヒト動物抗体のアミノ酸配列のいずれでもよいが、ヒト抗体のアミノ酸配列のC κまたはC λが好ましい。

[0053] 本発明の抗体は配列番号2で表されるヒトC R T H 2のアミノ酸配列の192番目のグリシンおよび194番目のアスパラギン酸の少なくとも一方のアミノ酸残基を認識し、結合する抗体である。

[0054] 本発明の抗体として具体的には、下記の(a)～(c)から選ばれる抗体が挙げられる。

(a) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の192番目のグリシンを認識し結合する抗体、

(b) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の194番目のアスパラギン酸を認識し結合する抗体、

(c) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の192番目のグリシンおよび194番目のアスパラギン酸の両方を認識し、結合する抗体。

[0055] また、本発明の抗体としては、配列番号2で表されるヒトC R T H 2のアミノ酸配列の192番目のグリシンおよび194番目のアスパラギン酸の少なくとも1つのアミノ酸残基を認識し、かつ配列番号2で表されるヒトC R T H 2のアミノ酸配列の12番目のプロリン、14番目のロイシン、15番

目のグルタミン酸、１７７番目のアスパラギン酸、１７８番目のグリシン、１７９番目のアルギニン、１８０番目のイソロイシン、１８１番目のメチオニン、１８３番目のチロシン、１８４番目のチロシン、１８５番目のアスパラギン、１８７番目のロイシン、１８８番目のロイシン、１８９番目のロイシン、１９５番目のアルギニン、１９６番目のアスパラギン酸および１９８番目のスレオニンからなる群から選ばれるアミノ酸残基の少なくとも１つを認識し、結合する抗体が挙げられる。

[0056] また、本発明の抗体としては配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１９２番目のグリシンおよび１９４番目のアスパラギン酸の少なくとも１つのアミノ酸残基を認識し、かつ下記の（ａ）～（ｇ）の少なくともいずれか１つを認識して結合する抗体が挙げられる。

（ａ）配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１２番目のプロリン、１４番目のロイシンおよび１５番目のグルタミン酸、

（ｂ）配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１７７番目のアスパラギン酸、１７８番目のグリシン、および１７９番目のアルギニン、

（ｃ）配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１８０番目のイソロイシンおよび１８１番目のメチオニン、

（ｄ）配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１８３番目のチロシン、１８４番目のチロシンおよび１８５番目のアスパラギン、

（ｅ）配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１８７番目のロイシン、１８８番目のロイシンおよび１８９番目のロイシン、

（ｆ）配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１９５番目のアルギニン、および

（ｇ）配列番号２で表されるヒトＣＲＴＨ２のアミノ酸配列の１９６番目のアスパラギン酸および１９８番目のスレオニン。

[0057] また本発明の抗体として、具体的には、下記の（ａ）～（ｄ）から選ばれる抗体が挙げられる。

（ａ）ＶＨの相補性決定領域（*complementary determ*

ining region; CDR、以下CDRと略記する) 1～3のアミノ酸配列が、それぞれ配列番号20、21および22で表されるアミノ酸配列を含み、かつVLのCDR1～3のアミノ酸配列が、それぞれ配列番号23、24および25で表されるアミノ酸配列を含む抗体、

(b) 前記(a)に記載の抗体と競合してヒトCRTH2に結合する抗体、

(c) 前記(a)に記載の抗体が結合するエピトープを含むエピトープに結合する抗体、および

(d) 前記(a)に記載の抗体が結合するエピトープと同じエピトープに結合する抗体。

[0058] 本発明の上記(b)の抗体とは、上記(a)の抗体とヒトCRTH2との結合を阻害する抗ヒトCRTH2抗体のことを示す。また、本発明の上記(c)の抗体とは、上記(a)に記載の抗体を第1抗体、および第1抗体が結合するエピトープを第1エピトープとした場合、当該第1エピトープを含むエピトープに結合する抗体のことを示す。

[0059] また本発明の抗体として、具体的には、下記の(a)～(c)から選ばれる抗体が挙げられる。

(a) 配列番号49で表されるアミノ酸配列または配列番号49で表されるアミノ酸配列中の18番目のロイシンをメチオニンに、77番目のアスパラギンをセリンに、93番目のバリンをスレオニンに、および117番目のスレオニンをバリンに置換する改変から選ばれる少なくとも1つの改変が導入されたアミノ酸配列を含むVHおよび配列番号33で表されるアミノ酸配列または配列番号33で表されるアミノ酸配列中の2番目のイソロイシンをバリンに、4番目のメチオニンをロイシンに、15番目のプロリンをロイシンに、および85番目のアラニンをプロリンに置換する改変から選ばれる少なくとも1つの改変が導入されたアミノ酸配列を含むVLを含む抗体、

(b) 配列番号49、51、53、55、57および59で表されるアミノ酸配列のいずれか1つを含むVH並びに配列番号33、35、37、39、41、43、45および47で表されるアミノ酸配列のいずれか1つを含む

V Lを含む抗体、並びに

(c) 配列番号17で表されるアミノ酸配列を含むV Hおよび配列番号19で表されるアミノ酸配列を含むV Lを含む抗体。

[0060] 上記(b)の抗体として、好ましくは以下の(1)～(3)から選ばれる抗体が挙げられる。

(1) 配列番号49で表されるアミノ酸配列を含むV H並びに配列番号33、35、37、39、41、43、45および47で表されるアミノ酸配列のいずれか1つを含むV Lを含む抗体、

(2) 配列番号59で表されるアミノ酸配列を含むV H並びに配列番号33、35、37、39、41、43、45および47で表されるアミノ酸配列のいずれか1つを含むV Lを含む抗体、並びに

(3) 配列番号51、53、55および57で表されるアミノ酸配列のいずれか1つを含むV H並びに配列番号33で表されるアミノ酸配列を含むV Lを含む抗体。

上記(b)の抗体として、特に好ましくは、配列番号51で表されるアミノ酸配列を含むV Hおよび配列番号33で表されるアミノ酸配列を含むV Lを含む抗体が挙げられる。

[0061] 本発明の抗体としては、ヒトC R T H 2の192番目のグリシンおよび194番目のアスパラギン酸の少なくとも一方をアラニンに置換したアミノ酸置換体に対し反応性が消失する抗体が挙げられる。

[0062] また、本発明の抗体には、ヒトC R T H 2のリガンド存在下で、ヒトC R T H 2への反応性が低下しない抗体が包含される。ヒトC R T H 2のリガンド存在下でヒトC R T H 2への反応性が低下しない抗体は、ヒトC R T H 2への反応性が低下する抗体に比べて、炎症局所のようにヒトC R T H 2のリガンドが高濃度で存在する条件下でも、高い反応性を示すことができる。従って、ヒトC R T H 2リガンド非依存的にヒトC R T H 2へ特異的に結合することができ、薬効を発揮することができる。

[0063] 本発明において、ヒトC R T H 2のリガンド存在下で抗体の反応性が低下

するとは、ヒトC R T H 2のリガンド非存在下でのヒトC R T H 2発現細胞に対する抗体の反応性と比較して、ヒトC R T H 2のリガンド存在下での該反応性が少なくとも5%以上低下することを示す。より厳密には10%以上低下することを示す。

[0064] ヒトC R T H 2のリガンドとしては、ヒトC R T H 2に特異的に結合するものであればいずれも包含されるが、好ましくはP G D 2またはD K P G D 2が挙げられる。より好ましくはD K P G D 2が挙げられる。

本発明において、活性化状態または不活性化状態のヒトC R T H 2に対する反応性が変化しないとは、グアノシン二リン酸（G D P）もしくはG D Pアナログまたはグアノシン三リン酸（G T P）もしくはG T Pアナログ存在下と非存在下で、抗体の、ヒトC R T H 2に対する反応性が変化しないことをいう。

G D Pアナログとしては例えばグアノシン 5'-0-(β -チオ)二リン酸（G D P β S）が挙げられる。G T Pアナログとしては、例えばグアノシン 5'-0-(γ -チオ)三リン酸（G T P γ S）が挙げられる。

[0065] 本発明の抗体には、中和活性を有しない抗体、アゴニスト活性を有しない抗体、ヒトC R T H 2のリガンドによるシグナルを増強しない抗体、または活性化状態または不活性化状態のヒトC R T H 2に対する反応性が変化しない抗体が包含される。

[0066] 本発明において、抗体の中和活性とは、抗体が持つヒトC R T H 2の生物活性を阻害する活性のことをいう。例えば、ヒトC R T H 2とそのリガンドとの結合を阻害する活性や、ヒトC R T H 2によるシグナル伝達を阻害する活性などのアンタゴニスト活性をいう。

[0067] 本発明において、アゴニスト活性とは、ヒトC R T H 2のリガンドの生物学的活性を模倣する活性をいい、C R T H 2の活性化及び、活性化に伴う種々の反応を誘導する活性をいう。本発明におけるアゴニスト活性として、具体的には細胞遊走活性、細胞の形態変化誘導活性などが挙げられる。

[0068] 本発明において、ヒトC R T H 2のリガンドによるシグナルとは、ヒトC

R T H 2 のリガンドがヒト C R T H 2 に結合し、ヒト C R T H 2 を活性化させることに伴うシグナルのことをいう。

[0069] ヒト C R T H 2 のリガンドによるシグナルおよびアゴニスト活性は、ヒト C R T H 2 の活性化に伴う種々の反応を解析することにより評価できる。例えば、ヒト C R T H 2 発現細胞の形態変化を解析することにより評価できる。

[0070] ヒト C R T H 2 発現細胞としては、ヒト C R T H 2 を発現していればいずれの細胞でもよいが、例えば、好酸球、好塩基球、T h 2 細胞、2 型自然リンパ球 (I L C 2) 、 *non classical monocyte*、T h 2 / T h 1 7 細胞などが挙げられる。

[0071] 本発明において、抗体がヒト C R T H 2 のリガンドによるシグナルを増強しないとは、ヒト C R T H 2 に対し、ヒト C R T H 2 のリガンドを単独で作用させたときと比べ、ヒト C R T H 2 のリガンドと抗体を共に作用させたときに、ヒト C R T H 2 の活性化及び、該活性化に伴う種々の反応を増強しないことを指す。

[0072] 本発明の抗体には、ヒト C R T H 2 発現細胞に対し細胞傷害活性を示す抗体が含まれる。本発明における細胞傷害活性としては、補体依存性細胞傷害活性（以下、C D C 活性と表記する）あるいは抗体依存性細胞傷害活性（以下、A D C C 活性と表記する）などが挙げられる。

[0073] 本発明における C D C 活性としては、細胞表面上のヒト C R T H 2 に結合した抗体分子が、F c 部分を介して補体系の C 1 q に結合し、その結果、C 1 から C 9 の各補体成分が活性化し、最終的には C 5 から C 9 が膜侵襲複合体と呼ばれる孔形成重合体を細胞膜上で形成して細胞溶解を引き起こす反応が挙げられる [Immunol Today, 1999 Dec;20(12):576-82.] 。

[0074] 本発明における A D C C 活性としては、細胞表面上のヒト C R T H 2 に結合した抗体分子が、F c 部分を介して、F c 受容体を発現した、例えば、ナチュラルキラー細胞（以下、N K 細胞と表記する）などを活性化することによる、パーフォリンやグランザイムなどの細胞傷害性分子の放出や貪食作用

の亢進などによって生じる細胞傷害反応が挙げられる [Chemical Immunology, 65, 88 (1997); Immunol Today, 20, 576 (1999)]。

[0075] 本発明の抗体にはマスト細胞に対する細胞傷害性を有しない抗体が含まれる。このような抗体は、マスト細胞の傷害に起因する炎症性メディエーターの遊離による副作用の懸念が無いという利点を有する。

[0076] 本発明の抗体には抗体のFc領域にN-グリコシド結合糖鎖が結合し、該N-グリコシド結合糖鎖の還元末端のN-アセチルグルコサミンにフコースが結合していない抗体が包含される。抗体のFc領域にN-グリコシド結合糖鎖が結合し、該N-グリコシド結合糖鎖の還元末端のN-アセチルグルコサミンにフコースが結合していない抗体としては、例えば $\alpha 1, 6$ -フコース転移酵素遺伝子が欠損したCHO細胞（国際公開第2005/035586号、国際公開第02/31140号）を用いて作製される抗体が挙げられる。抗体のFc領域にN-グリコシド結合糖鎖が結合し、該N-グリコシド結合糖鎖の還元末端のN-アセチルグルコサミンにフコースが結合していない本発明の抗体は、高いADCC活性を有する。

[0077] 本発明の抗体には抗体のFc領域のアミノ酸残基を、Fc受容体との結合活性が高くなるように改変した抗体が包含される。抗体のFc領域のアミノ酸残基を、Fc受容体との結合活性が高くなるように改変した抗体としては、例えば米国特許第7317091号明細書記載の方法で作製された抗体分子を挙げることができる。

[0078] 本発明の抗体には、抗体の可変領域を含むポリペプチドの表面電荷や早期エンドソーム内でのpHにおける抗原結合活性を改変し血中半減期が伸びた抗体が包含される。

[0079] 抗体分子の可変領域を含むポリペプチドの表面電荷や早期エンドソーム内でのpHにおける抗原結合活性を改変し血中半減期が延びた抗体としては、例えば日本国特開2013-165716号公報、日本国特開2012-021004号公報記載の方法で作製された抗体を挙げることができる。

[0080] 本発明の抗体には、ヒト型キメラ抗体（以下、単にキメラ抗体とも表記す

る)、ヒト型CDR移植抗体(以下ヒト化抗体とも表記する)およびヒト抗体などの遺伝子組換え抗体が包含される。

[0081] キメラ抗体は、ヒト以外の動物(非ヒト動物)の抗体のVHおよびVLとヒト抗体のCHとおよびCLからなる抗体をいう。非ヒト動物としては、マウス、ラット、ハムスター、ラビット等、ハイブリドーマを作製することが可能であれば、いかなるものも用いることができる。

[0082] 本発明のキメラ抗体は、ヒトCRTH2に特異的に反応するヒト以外の動物の抗体のVHおよびVLをコードするcDNAを取得し、ヒト抗体のCHおよびCLをコードする遺伝子を有する動物細胞用発現ベクターにそれぞれ挿入してキメラ抗体発現ベクターを構築し、動物細胞へ導入することにより発現させ、製造することができる。

[0083] ヒト化抗体は、ヒト以外の動物の抗体のVHおよびVLのCDRをヒト抗体のVHおよびVLの適切な位置に移植した抗体を意味する。

[0084] 本発明のヒト化抗体は、ヒトCRTH2に特異的に反応するヒト以外の動物の抗体のVHおよびVLのCDRを任意のヒト抗体のVHおよびVLのフレームワーク(以下、FRと表記する)に移植した可変領域(以下V領域とも表記する)をコードするcDNAを構築し、CHおよびCLをコードするDNAを有する動物細胞用発現ベクターにそれぞれ挿入してヒト化抗体発現ベクターを構築し、該発現ベクターを動物細胞へ導入することにより発現させ、製造することができる。

[0085] ヒト抗体のVHおよびVLのFRのアミノ酸配列としては、ヒト抗体由来のアミノ酸配列であれば、いかなるものでも用いることができる。例えば、Protein Data Bankなどのデータベースに登録されているヒト抗体のVHおよびVLのFRのアミノ酸配列、またはヒト抗体のVHおよびVLのFRの各サブグループの共通アミノ酸配列(Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services, 1991)などが挙げられる。

[0086] これらのアミノ酸配列において、1以上のアミノ酸が欠失、付加、置換ま

たは挿入され、かつヒトC R T H 2と特異的に結合して、かつ、例えば、細胞傷害活性等の生物活性において同等の機能を有する抗体または該抗体断片も本発明の抗体に包含される。

[0087] 本発明の抗体にはサルC R T H 2に結合する抗体が包含される。サルC R T H 2としては例えばマーモセットC R T H 2、カニクイザルC R T H 2およびアカゲザルC R T H 2が挙げられる。好ましくはカニクイザルC R T H 2が挙げられる。

[0088] 本発明の抗体としては、F cと抗体断片とが結合したF c融合タンパク質、F cと天然に存在するリガンドまたは受容体とが結合したF c融合タンパク質（イムノアドヘシンともいう）、複数のF c領域を融合させたF c融合タンパク質等も本発明に包含される。また、抗体を安定化させるためまたは血中半減期を制御するためにアミノ酸残基置換を行ったアミノ酸残基改変を含む改変F c領域なども本発明の抗体に用いることができる。

[0089] 本発明において、抗体断片とは、配列番号2で表されるヒトC R T H 2のアミノ酸配列の192番目のグリシンおよび194番目のアスパラギン酸の少なくとも一方を認識し、結合する抗原結合ドメインを含み、且つ抗原結合活性を有する断片である。本発明の抗体断片としては、F a b、F a b'、F (a b')₂、single chain F v（以下、scF vと記す）、d i a b o d y、d s F vおよび複数のC D Rを含むペプチドなどが挙げられる。

[0090] F a bは、I g Gを蛋白質分解酵素パパインで処理して得られる断片のうち（H鎖の224番目のアミノ酸残基で切断される）、H鎖のN末端側約半分とL鎖全体がジスルフィド結合で結合した分子量約5万の抗原結合活性を有する抗体断片である。

[0091] 本発明のF a bは、本発明のヒトC R T H 2に特異的に結合する抗体を蛋白質分解酵素パパインで処理して得ることができる。または、該抗体のF a bをコードするD N Aを原核生物用発現ベクターあるいは真核生物用発現ベクターに挿入し、該ベクターを原核生物あるいは真核生物へ導入することに

より発現させ、製造することができる。

[0092] $F(a b')_2$ は、IgGを蛋白質分解酵素ペプシンで処理して得られる断片のうち(H鎖の234番目のアミノ酸残基で切断される)、Fabがヒンジ領域のジスルフィド結合を介して結合されたものよりやや大きい、分子量約10万の抗原結合活性を有する抗体断片である。

[0093] 本発明の $F(a b')_2$ は、本発明のヒトC R T H 2に特異的に結合する抗体を蛋白質分解酵素ペプシンで処理して得ることができる。または、下記のFab'をチオエーテル結合あるいはジスルフィド結合させ、作製することができる。

[0094] Fab'は、上記 $F(a b')_2$ のヒンジ領域のジスルフィド結合を切断した分子量約5万の抗原結合活性を有する抗体断片である。

本発明のFab'は、本発明のヒトC R T H 2に特異的に結合する $F(a b')_2$ 組成物を還元剤ジチオスレイトール処理して得ることができる。または、該抗体のFab'断片をコードするDNAを原核生物用発現ベクターあるいは真核生物用発現ベクターに挿入し、該ベクターを原核生物あるいは真核生物へ導入することにより発現させ、製造することができる。

[0095] scFvは、1本のVHと1本のVLとを4個のGlyおよび1個のSer残基からなるリンカー(G4S)を任意の個数つなげたリンカーペプチドなどの適当なペプチドリリンカー(P)を用いて連結した、VH-P-VLないしはVL-P-VHポリペプチドで、抗原結合活性を有する抗体断片である。

[0096] 本発明のscFvは、本発明のヒトC R T H 2に特異的に結合する抗体のVHおよびVLをコードするcDNAを取得し、scFvをコードするDNAを構築し、該DNAを原核生物用発現ベクターあるいは真核生物用発現ベクターに挿入し、該発現ベクターを原核生物あるいは真核生物へ導入することにより発現させ、製造することができる。

[0097] d i a b o d yは、scFvが二量体化した抗体断片で、二価の抗原結合活性を有する抗体断片である。二価の抗原結合活性は、同一であることもで

きるし、一方を異なる抗原結合活性とすることもできる。

[0098] 本発明の *d i a b o d y* は、本発明のヒト C R T H 2 に特異的に結合する抗体の V H および V L をコードする c D N A を取得し、s c F v をコードする D N A を P のアミノ酸配列の長さが 8 残基以下となるように構築し、該 D N A を原核生物用発現ベクターまたは真核生物用発現ベクターに挿入し、該発現ベクターを原核生物または真核生物へ導入することにより発現させ、製造することができる。

[0099] d s F v は、V H および V L 中のそれぞれ 1 アミノ酸残基をシステイン残基に置換したポリペプチドを該システイン残基間のジスルフィド結合を介して結合させたものをいう。システイン残基に置換するアミノ酸残基は R e i t e r らにより示された方法 (Protein Engineering, 7, 697-704, 1994) に従って、抗体の立体構造予測に基づいて選択することができる。

[0100] 本発明の d s F v は、本発明のヒト C R T H 2 に特異的に結合する抗体の V H および V L をコードする c D N A を取得し、d s F v をコードする D N A を構築し、該 D N A を原核生物用発現ベクターまたは真核生物用発現ベクターに挿入し、該発現ベクターを原核生物または真核生物へ導入することにより発現させ、製造することができる。

[0101] C D R を含むペプチドは、V H または V L の C D R の少なくとも 1 領域以上を含んで構成される。複数の C D R を含むペプチドは、直接または適当なペプチドリンカーを介して結合させることができる。

[0102] 本発明の C D R を含むペプチドは、本発明のヒト C R T H 2 に特異的に結合する抗体の V H および V L の C D R をコードする D N A を構築し、該 D N A を原核生物用発現ベクターあるいは真核生物用発現ベクターに挿入し、該発現ベクターを原核生物または真核生物へ導入することにより発現させ、製造することができる。

[0103] また、C D R を含むペプチドは、F m o c 法 (フルオレニルメチルオキシカルボニル法)、t B o c 法 (t-ブチルオキシカルボニル法) などの化学合成法によって製造することもできる。

[0104] 上述の抗体または該抗体断片を構成するアミノ酸配列において、1つ以上のアミノ酸が欠失、置換、挿入または付加され、かつ上述の抗体または該抗体断片と同様な活性を有するモノクローナル抗体または該抗体断片も、本発明の抗体または該抗体断片に包含される。欠失、置換、挿入または付加されるアミノ酸の数は1個以上でありその数は特に限定されないが、モレキュラー・クローニング第2版、カレント・プロトコールズ・イン・モレキュラー・バイオロジー、Nucleic Acids Research, 10, 6487 (1982)、Proc. Natl. Acad. Sci., USA, 79, 6409 (1982)、Gene, 34, 315 (1985)、Nucleic Acids Research, 13, 4431 (1985)、Proc. Natl. Acad. Sci. USA, 82, 488 (1985)等に記載の部位特異的変異導入法等の周知の技術により、欠失、置換もしくは付加できる程度の数であり、例えば、1～数十個、好ましくは1～20個、より好ましくは1～10個、さらに好ましくは1～5個である。

[0105] 本発明のヒトC R T H 2または抗体のアミノ酸配列において1以上のアミノ酸残基が欠失、置換、挿入または付加されたとは、同一配列中の任意かつ1もしくは複数のアミノ酸配列中において、1または複数のアミノ酸残基の欠失、置換、挿入または付加があることを意味し、欠失、置換、挿入または付加が同時に生じてもよく、置換、挿入または付加されるアミノ酸残基は天然型と非天然型とを問わない。

[0106] 天然型アミノ酸残基としては、例えば、L-アラニン、L-アスパラギン、L-アスパラギン酸、L-グルタミン、L-グルタミン酸、グリシン、L-ヒスチジン、L-イソロイシン、L-ロイシン、L-リジン、L-メチオニン、L-フェニルアラニン、L-プロリン、L-セリン、L-スレオニン、L-トリプトファン、L-チロシン、L-バリン、L-システインなどが挙げられる。

[0107] 以下に、相互に置換可能なアミノ酸残基の好ましい例を示す。同一群に含まれるアミノ酸残基は相互に置換可能である。

A群：ロイシン、イソロイシン、ノルロイシン、バリン、ノルバリン、アラニン、2-アミノブタン酸、メチオニン、O-メチルセリン、t-ブチルグ

リシン、 ϵ -ブチルアラニン、シクロヘキシルアラニン

B群：アスパラギン酸、グルタミン酸、イソアスパラギン酸、イソグルタミン酸、2-アミノアジピン酸、2-アミノスベリン酸

C群：アスパラギン、グルタミン

D群：リジン、アルギニン、オルニチン、2, 4-ジアミノブタン酸、2, 3-ジアミノプロピオン酸

E群：プロリン、3-ヒドロキシプロリン、4-ヒドロキシプロリン

F群：セリン、スレオニン、ホモセリン

G群：フェニルアラニン、チロシン

[0108] 本発明の形質転換体としては、ヒトC R T H 2に特異的に結合する抗体分子をコードするDNAを宿主細胞に導入して得られる形質転換体であって、本発明の抗体を生産する形質転換体であればいかなる形質転換体でも包含される。具体的な例としては、ヒトC R T H 2に特異的に結合する抗体分子をコードするDNAを以下の(a)～(i)などの宿主細胞に導入して得られる形質転換体が好例として挙げられる。

(a) チャイニーズハムスター卵巣組織由来CHO細胞；

(b) ラットミエローマ細胞株Y B 2 / 3 H L . P 2 . G 1 1 . 1 6 A g . 2 0 細胞；

(c) マウスミエローマ細胞株N S O 細胞；

(d) マウスミエローマ細胞株S P 2 / O - A g 1 4 細胞；

(e) シリアンハムスター腎臓組織由来B H K 細胞；

(f) 抗体を生産するハイブリドーマ細胞；

(g) ヒト白血病細胞株ナマルバ細胞；

(h) 胚性幹細胞；

(i) 受精卵細胞。

[0109] また、抗体のFc領域にN-グリコシド結合糖鎖が結合し、該N-グリコシド結合糖鎖の還元末端のN-アセチルグルコサミンにフコースが結合していない抗体を生産する本発明の形質転換体としては、ヒトC R T H 2に特異

的に結合する抗体分子をコードするDNAを国際公開第2005/035586号、国際公開第02/31140号に記載の方法で作製された糖転移酵素の低下または欠損した宿主細胞に導入して得られる形質転換体が好例として挙げられる。

[0110] 本発明の抗体または該抗体断片の製造法としては、本発明の抗体または該抗体断片を生産する形質転換体を培養する製造方法であれば、いかなる抗体製造方法でも包含されるが、好例として、本発明の抗体または該抗体断片を生産する形質転換体を培養し、培養物中に抗体または該抗体断片を生成蓄積させ、該抗体または該抗体断片を採取し、精製する抗体または該抗体断片の製造方法が挙げられる。

[0111] 上記製造法により製造される抗体または該抗体断片も、本発明の抗体または該抗体断片として挙げられる。

[0112] 本発明の組成物としては、本発明の抗体または該抗体断片を含む組成物であればいずれの組成物であってもよく、抗体に結合する糖鎖が単一の抗体分子を含む組成物、または複数の糖鎖構造を有する抗体分子を含む組成物であってもよい。また、適当な添加剤、バッファー等を含む組成物であってもよい。本発明の組成物としては、好ましくは本発明の抗体または該抗体断片を有効成分として含有する医薬、診断薬等が挙げられる。

[0113] 本発明の医薬または診断薬としては、本発明の抗体または該抗体断片を有効成分として含有する医薬または診断薬であればいかなる医薬または診断薬も含有される。好例として、ヒトC R T H 2 発現細胞に関連した疾患の医薬または診断薬が挙げられる。

[0114] 本発明の治療方法としては、本発明の抗体または該抗体断片を有効量投与する治療方法であればいかなる治療方法も含まれるが、好ましくは、ヒトC R T H 2 発現細胞に関連した疾患の治療方法が挙げられる。

[0115] 本発明の抗体または該抗体断片の使用としては、ヒトC R T H 2 発現細胞に関連した疾患の治療薬を製造するための本発明の抗体または該抗体断片の使用であれば、いかなる抗体または該抗体断片の使用も含まれる。また、本

発明の抗体または該抗体断片はヒトC R T H 2 発現細胞が関係する障害または疾患の治療および予防の少なくとも一方に用いることができる。

[0116] ヒトC R T H 2 発現細胞が関係する障害または疾患としては、限定するものではないが、例えば、アレルギー性疾患、自己免疫疾患、好酸球増多および機能亢進の少なくとも一方を伴う疾患、T h 2 細胞の増多および機能亢進の少なくとも一方を伴う疾患並びにI L C 2 の増多および機能亢進の少なくとも一方を伴う疾患などが挙げられる。

[0117] より具体的には、例えば、アレルギー性又は非アレルギー性鼻炎又は副鼻腔炎、慢性副鼻腔炎又は鼻炎、鼻ポリープ、鼻ポリープを伴う慢性副鼻腔炎、抗酸球性副鼻腔炎、急性副鼻腔炎、喘息、小児喘息、アレルギー性気管支炎、肺炎、農夫の疾患 (Farmer's disease)、過敏性気道、感染、例えば、細菌又はウイルス又は蠕虫又は真菌又は原生動物その他の病原に起因するアレルギー性結膜炎、気管支炎又は肺臓炎、気管支拡張症、成人呼吸促迫症候群、気管支及び肺浮腫、様々な起源、例えば毒ガス、蒸気の吸引、吸入に起因する気管支炎又は肺臓炎又は間質性肺臓炎、心不全、X線、放射線、化学療法に起因する気管支炎又は肺臓炎又は間質性肺臓炎、膠原病、例えばエリテマトーデス、全身性強皮症に関連する気管支炎又は肺臓炎又は間質性肺臓炎、肺線維症、特発性肺線維症 (I P F)、様々な起源の間質性肺疾患又は間質性肺臓炎、例えば、石綿肺症、珪肺症、m. B o e c k 又はサルコイドーシス、肉芽腫症、嚢胞性線維症又はムコビシドーシス、又は $\alpha 1$ -抗トリプシン欠乏症、好酸球性セルライト (例えば、W e i l 症候群)、好酸球性肺炎 (例えば、L o e f f l e r 症候群、慢性好酸球性肺炎)、好酸球性筋膜炎 (例えば、S h u l m a n 症候群)、抗酸球性食道炎、抗酸球増加症候群、遅延型過敏、非アレルギー性喘息、運動誘発性気管支収縮；慢性閉塞性肺疾患 (C O P D)、急性気管支炎、慢性気管支炎、肺気腫；全身性アナフィラキシー又は過敏性反応、薬物アレルギー (例えば、ペニシリン、セファロスポリンに対して)、汚染トリプトファンの摂取による好酸球増多筋痛症候群、昆虫刺傷アレルギー；自己免疫疾患、例えば関節

リウマチ、乾癬性関節炎、多発性硬化症、全身性エリテマトーデス、重症筋無力症、免疫性血小板減少症（成人ITP、新生児血小板減少症、小児ITP）、免疫性溶血性貧血（自己免疫及び薬物誘発）、Evans症候群（血小板及び赤血球免疫性血球減少症）、新生児のRh疾患、Goodpasture症候群（抗GBM疾患）、セリアック病（Celiac）、自己免疫性心筋症、若年発症糖尿病；糸球体腎炎、自己免疫性甲状腺炎、ベーチェット病；移植片拒絶（例えば、移植において）、例えば、同種移植片拒絶又は移植片対宿主病；炎症性腸疾患、例えばクローン病及び潰瘍性大腸炎；脊椎関節症；強皮症；乾癬（T細胞媒介乾癬を含めて）及び炎症性皮肤病、例えば、皮膚炎、湿疹、アトピー性皮膚炎、アレルギー性接触皮膚炎、蕁麻疹（例えば、慢性突発性、慢性自発性、物理的蕁麻疹）、水疱性類天疱瘡；血管炎（例えば、壊死性、皮膚性、肉芽腫性及び過敏性血管炎、抗酸球性多発血管炎性肉芽腫症）；結節性紅斑；好酸球性筋炎、好酸球性筋膜炎、皮膚又は器官の白血球浸潤を伴う癌を含めた炎症性又はアレルギー性疾患及び状態が挙げられる。

[0118] ヒトCRTH2発現細胞が関係する障害または疾患として、好ましくは喘息、小児喘息、慢性閉塞性肺疾患、アトピー性皮膚炎、アレルギー性鼻炎および急性または慢性副鼻腔炎が挙げられる。

本発明の抗体もしくは該抗体断片、又はこれらの誘導体を含有する医薬は、有効成分としての該抗体もしくは該抗体断片、又はこれらの誘導体のみを含むものであってもよいが、通常は薬理学的に許容される1以上の担体と一緒に混合し、製剤学の技術分野において公知の方法により製造した医薬製剤として提供される。

[0119] 投与経路としては、例えば、経口投与、又は口腔内、気道内、直腸内、皮下、筋肉内、静脈内、若しくは腹腔内などの非経口投与が挙げられる。投与形態としては、例えば、噴霧剤、カプセル剤、錠剤、散剤、顆粒剤、シロップ剤、乳剤、坐剤、注射剤、軟膏、又はテープ剤などが挙げられる。

[0120] 経口投与に適当な製剤としては、例えば、乳剤、シロップ剤、カプセル剤

、錠剤、散剤、又は顆粒剤などで挙げられる。

[0121] 本発明の抗体には、本発明の抗体またはその抗体断片に放射性同位元素、低分子の薬剤、高分子の薬剤、蛋白質、核酸などを化学的または遺伝子工学的に結合させた抗体の誘導体を包含する。

[0122] 抗体の誘導体を治療方法、予防方法、治療薬または治療薬として使用する場合には、本発明の抗体またはその抗体断片に結合する薬剤として、化学療法剤、抗体医薬、免疫賦活剤、高分子の薬剤などが挙げられる。タンパク質としては、サイトカイン、増殖因子、毒素タンパク質などが挙げられる。核酸としては、デコイ、アンチセンス、*siRNA*、*miRNA*などが挙げられる。

抗体の誘導体を検出方法、定量方法、検出用試薬、または定量用試薬として使用する場合には、本発明の抗体またはその抗体断片に結合する薬剤として、通常の免疫学的検出または測定法で用いられる標識体が挙げられる。

[0123] 本発明における、抗体の誘導体は、本発明の抗体またはその抗体断片のH鎖またはL鎖のN末端側またはC末端側、抗体またはその抗体断片中の適当な置換基または側鎖、さらには抗体または該抗体断片中の糖鎖などに、放射性同位元素、低分子の薬剤、高分子の薬剤、蛋白質などを化学的手法〔抗体工学入門、地人書館（1994）記載の方法など〕により結合させることにより製造することができる。

[0124] また、本発明における、抗体の誘導体は、本発明の抗体または抗体断片をコードするDNAと、結合させたい蛋白質をコードするDNAを連結させて発現ベクターに挿入し、該発現ベクターを適当な宿主細胞へ導入し、発現させる遺伝子工学的手法より製造することができる。

[0125] 放射性同位元素としては、例えば、 ^{131}I 、 ^{125}I 、 ^{90}Y 、 ^{64}Cu 、 ^{99}Tc 、 ^{77}Lu 、または ^{211}At などが挙げられる。放射性同位元素は、クロラミンT法などによって抗体に直接結合させることができる。また、放射性同位元素をキレートする物質を抗体に結合させてもよい。キレート剤としては、例えば、1-イソチオシアネートベンジル-3-メチルジエチレントリアミ

ンペンタ酢酸（MX-DTPA）などが挙げられる。

[0126] 低分子の薬剤としては、例えば、アクリジニウムエステルもしくはロフィンなどの発光物質、またはフルオレセインイソチオシアネート（FITC）もしくはテトラメチルローダミンイソチオシアネート（RITC）などの蛍光物質などが挙げられる。

[0127] 低分子の薬剤と抗体とを結合させる方法としては、例えば、グルタルアルデヒドを介して薬剤と抗体のアミノ基間を結合させる方法、または水溶性カルボジイミドを介して薬剤のアミノ基と抗体のカルボキシル基を結合させる方法などが挙げられる。

[0128] 高分子の薬剤としては、例えば、ポリエチレングリコール（以下、PEGと表記する）、アルブミン、デキストラン、ポリオキシエチレン、スチレンマレイン酸コポリマー、ポリビニルピロリドン、ピランコポリマー、またはヒドロキシプロピルメタクリルアミドなどが挙げられる。

[0129] 免疫学的検出または測定方法とは、標識を施した抗原または抗体を用いて、抗体量または抗原量を検出または測定する方法である。免疫学的検出または測定方法としては、放射性物質標識免疫抗体法（RIA）、酵素免疫測定法（EIAまたはELISA）、蛍光免疫測定法（FIA）、発光免疫測定法（luminescent immunoassay）、ウエスタンブロット法または物理化学的手法などが挙げられる。

[0130] 本発明の抗体または該抗体断片を用いて、上記の方法に従いヒトCPTH2が発現した細胞を検出または測定することにより、ヒトCPTH2に関連する疾患を診断することができる。

[0131] 本発明においてヒトCPTH2を検出または測定する対象となる生体試料としては、組織細胞、血液、血漿、血清、唾液、尿、糞便、組織液、肺胞洗浄液または培養液など、細胞外に分泌されたヒトCPTH2もしくはその一部を含むペプチド断片、又はヒトCPTH2を発現している細胞を含む可能性のあるものであれば特に限定されない。

[0132] 本発明の抗体若しくはその抗体断片、またはこれらの誘導体を含有する診

断薬は、目的の診断法に応じて、抗原抗体反応を行なうための試薬、該反応の検出用試薬を含んでもよい。抗原抗体反応を行なうための試薬としては、緩衝剤、塩などが挙げられる。検出用試薬としては、該抗体若しくはその抗体断片、またはこれらの誘導体を認識する標識された二次抗体、または標識に対応した基質などの通常の免疫学的検出または測定法に用いられる試薬が挙げられる。

[0133] 以下に、本発明の抗体の製造方法、疾患の治療方法、及び疾患の診断方法について、具体的に説明する。

[0134] 1. 抗体の製造方法

(1) 抗原の調製

抗原となるヒトC R T H 2又はヒトC R T H 2を発現させた細胞は、ヒトC R T H 2全長又はその部分長をコードするc D N Aを含む発現ベクターを、大腸菌、酵母、昆虫細胞、又は動物細胞などに導入することにより、得ることができる。

[0135] また、ヒトC R T H 2を多量に発現している各種ヒト培養細胞、ヒト組織などからヒトC R T H 2を精製することによっても、得ることができる。また、該培養細胞、又は該組織などをそのまま抗原として用いることもできる。さらに、F m o c法、又はt B o c法などの化学合成法によりヒトC R T H 2の部分配列を有する合成ペプチドを調製し、抗原に用いることもできる。

[0136] ヒトC R T H 2またはヒトC R T H 2の部分配列を有する合成ペプチドには、C末端もしくはN末端にF L A GもしくはH i sなどの公知のタグが付加されていてもよい。

[0137] 本発明で用いられるヒトC R T H 2は、Molecular Cloning、A Laboratory Manual、Second Edition、Cold Spring Harbor Laboratory Press (1989)又はCurrent Protocols in molecular Biology、John Wiley & Sons (1987-1997)などに記載された方法などを用い、例えば以下の方法により、該ヒトC R T H 2をコードするD N Aを宿主細胞中で発現させて、製造することができ

る。

[0138] まず、ヒトC R T H 2をコードする部分を含む完全長c D N Aを適当な発現ベクターのプロモーターの下流に挿入することにより、組換えベクターを作製する。上記完全長c D N Aの代わりに、完全長c D N Aをもとにして調製された、ポリペプチドをコードする部分を含む適当な長さのD N A断片を用いてもよい。次に、得られる該組換えベクターを、該発現ベクターに適した宿主細胞に導入することにより、ポリペプチドを生産する形質転換体を得ることができる。

[0139] 発現ベクターとしては、使用する宿主細胞における自律複製又は染色体中への組込みが可能で、ポリペプチドをコードするD N Aを転写できる位置に、適当なプロモーターを含有しているものであればいずれも用いることができる。

[0140] 宿主細胞としては、大腸菌などのエシェリヒア属などに属する微生物、酵母、昆虫細胞、又は動物細胞など、目的とする遺伝子を発現できるものであればいずれも用いることができる。

[0141] 大腸菌などの原核生物を宿主細胞として用いる場合、組換えベクターは、原核生物中で自律複製が可能であると同時に、プロモーター、リボソーム結合配列、ヒトC R T H 2をコードする部分を含むD N A、及び転写終結配列を含むベクターであることが好ましい。

[0142] また、該組換えベクターには、転写終結配列は必ずしも必要ではないが、構造遺伝子の直下に転写終結配列を配置することが好ましい。さらに、該組換えベクターには、プロモーターを制御する遺伝子を含んでいてもよい。

[0143] 該組換えベクターとしては、リボソーム結合配列であるシャイン・ダルガルノ配列（S D配列ともいう）と開始コドンとの間を適当な距離（例えば6～18塩基）に調節したプラスミドを用いることが好ましい。

[0144] また、該ヒトC R T H 2をコードするD N Aの塩基配列としては、宿主内での発現に最適なコドンとなるように塩基を置換することができ、これにより目的とするヒトC R T H 2の生産率を向上させることができる

[0145] 発現ベクターとしては、使用する宿主細胞中で機能を発揮できるものであればいずれも用いることができ、例えば、pBTrp2、pBTac1、pBTac2（以上、ロシュ・ダイアグノスティックス社）、pKK233-2（ファルマシア社）、pSE280（インビトロジェン社）、pGEMEX-1（プロメガ社）、pQE-8（キアゲン社）、pKYP10（日本国特開昭58-110600号公報）、pKYP200 [Agricultural Biological Chemistry, 48, 669(1984)]、pLSA1 [Agric Biol. Chem., 53, 277(1989)]、pGEL1 [Proc. Natl. Acad. Sci. USA, 82, 4306 (1985)]、pBluescript IISK（-）（ストラタジーン社）、pTrs30 [大腸菌JM109/pTrs30 (FERM BP-5407) より調製]、pTrs32 [大腸菌JM109/pTrs32 (FERM BP-5408) より調製]、pGHA2 [大腸菌IGHA2 (FERM BP-400) より調製、日本国特開昭60-221091号公報]、pGKA2 [大腸菌IGKA2 (FERM BP6798) より調製、日本国特開昭60-221091号公報]、pTerm2（米国特許第4686191号明細書、米国特許第4939094号明細書、米国特許第5160735号明細書）、pSupex、pUB110、pTP5、pC194、pEG400 [J. Bacteriol., 172, 2392 (1990)]、pGEX（ファルマシア社）、pETシステム（ノバジェン社）、又はpME18SFL3などが挙げられる。

[0146] プロモーターとしては、使用する宿主細胞中で機能を発揮できるものであればいかなるものでもよい。例えば、trpプロモーター (Ptrp)、lacプロモーター、PLプロモーター、PRプロモーター、又はT7プロモーターなどの、大腸菌又はファージなどに由来するプロモーターを挙げることができる。また、Ptrpを2つ直列させたタンデムプロモーター、tacプロモーター、lacT7プロモーター、又はletIプロモーターなどの人為的に設計改変されたプロモーターなども用いることができる。

[0147] 宿主細胞としては、例えば、大腸菌XL-1Blue、大腸菌XL2-B

lue、大腸菌DH1、大腸菌MC1000、大腸菌KY3276、大腸菌W1485、大腸菌JM109、大腸菌HB101、大腸菌No. 49、大腸菌W3110、大腸菌NY49、又は大腸菌DH5 α などが挙げられる。

[0148] 宿主細胞への組換えベクターの導入方法としては、使用する宿主細胞へDNAを導入する方法であればいずれも用いることができ、例えば、カルシウムイオンを用いる方法 [Proc. Natl. Acad. Sci. USA, 69, 2110 (1972); Gene, 17, 107 (1982); Molecular & General Genetics, 168, 111 (1979)] が挙げられる。

[0149] 動物細胞を宿主として用いる場合、発現ベクターとしては、動物細胞中で機能を発揮できるものであればいずれも用いることができ、例えば、pcDNA1、pcDM8（フナコシ社）、pAGE107 [日本国特開平03-22979号公報；Cytotechnology, 3, 133 (1990)]、pAS3-3（日本国特開平02-227075号公報）、pcDM8 [Nature, 329, 840 (1987)]、pcDNA1/Amp（インビトロジェン社）、pcDNA3.1（インビトロジェン社）、pREP4（インビトロジェン社）、pAGE103 [J. Biochemistry, 101, 1307 (1987)]、pAGE210、pME18SFL3、pKANTEX93（国際公開第97/10354号）、N5KG1val（米国特許第6001358号明細書）、INPEP4（Biogen-IDEC社）およびトランスポゾンベクター（国際公開第2010/143698号）などが挙げられる。

[0150] プロモーターとしては、動物細胞中で機能を発揮できるものであればいずれも用いることができ、例えば、サイトメガロウイルス（CMV）のimmediate early（IE）遺伝子のプロモーター、SV40の初期プロモーター、レトロウイルスのプロモーター、メタロチオネインプロモーター、ヒートショックプロモーター、SR α プロモーター、又はモロニーマウス白血病ウイルスのプロモーター若しくはエンハンサーが挙げられる。また、ヒトCMVのIE遺伝子のエンハンサーをプロモーターと共に用いても

よい。

[0151] 宿主細胞としては、例えば、ヒト白血病細胞 N a m a l w a 細胞、サル細胞 C O S 細胞、チャイニーズハムスター卵巣細胞 C H O 細胞 [Journal of Experimental Medicine, 108, 945(1958); Proc. Natl. Acad. Sci. USA, 60, 1275(1968); Genetics, 55, 513(1968); Chromosoma, 41, 129(1973); Methods in Cell Science, 18, 115(1996); Radiation Research, 148, 260(1997); Proc. Natl. Acad. Sci. USA, 77, 4216(1980); Proc. Natl. Acad. Sci. USA, 60, 1275(1968); Cell, 6, 121(1975); Molecular Cell Genetics, Appendix I, II(pp.883-900)]、C H O / D G 4 4、C H O - K 1 (A T C C 番号: C C L - 6 1)、D U k X B 1 1 (A T C C 番号: C C L - 9 0 9 6)、P r o - 5 (A T C C 番号: C C L - 1 7 8 1)、C H O - S (L i f e T e c h n o l o g i e s、C a t # 1 1 6 1 9)、P r o - 3、ラットミエローマ細胞 Y B 2 / 3 H L. P 2. G 1 1. 1 6 A g. 2 0 (又は Y B 2 / 0 とともいう)、マウスミエローマ細胞 N S 0、マウスミエローマ細胞 S P 2 / 0 - A g 1 4、シリアンハムスター細胞 B H K 又は H B T 5 6 3 7 (日本国特開昭 6 3 - 0 0 0 2 9 9 号公報)などが挙げられる。

[0152] 宿主細胞への組換えベクターの導入方法としては、動物細胞に D N A を導入する方法であればいずれも用いることができる。例えば、エレクトロポレーション法 [Cytotechnology, 3, 133(1990)]、リン酸カルシウム法 (日本国特開平 0 2 - 2 2 7 0 7 5 号公報)、又はリポフェクション法 [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)]などが挙げられる。

[0153] 以上のようにして得られるヒト C R T H 2 をコードする D N A を組み込んだ組換えベクターを保有する微生物、又は動物細胞などの由来の形質転換体を培地に培養し、培養物中に該ヒト C R T H 2 を生成蓄積させ、該培養物から採取することにより、ヒト C R T H 2 を製造することができる。該形質転換体を培地に培養する方法は、宿主の培養に用いられる通常の方法に従って行うことができる。

[0154] 真核生物由来の細胞で発現させた場合には、糖又は糖鎖が付加されたヒト

C R T H 2を得ることができる。

- [0155] 誘導性のプロモーターを用いた組換えベクターで形質転換した微生物を培養するときには、必要に応じてインデューサーを培地に添加してもよい。例えば、*l a c*プロモーターを用いた組換えベクターで形質転換した微生物を培養する場合にはイソプロピル- β -D-チオガラクトピラノシドなどを、*t r p*プロモーターを用いた組換えベクターで形質転換した微生物を培養する場合にはインドールアクリル酸などを培地に添加してもよい。
- [0156] 動物細胞を宿主として得られる形質転換体を培養する培地としては、例えば、一般に使用されているRPMI 1640培地 [The Journal of the American Medical Association, 199, 519(1967)]、EagleのMEM培地 [Science, 122, 501(1952)]、ダルベッコ改変MEM培地 [Virology, 8, 396(1959)]、199培地 [Proc. Soc. Exp. Biol. Med., 73, 1(1950)]、Iscove's Modified Dulbecco's Medium (IMDM) 培地、又はこれら培地に牛胎児血清 (FBS) などを添加した培地などが挙げられる。培養は、通常pH 6~8、30~40℃、5%CO₂存在下などの条件下で1~7日間行う。また、培養中必要に応じて、カナマイシン又はペニシリンなどの抗生物質を培地に添加してもよい。
- [0157] ヒトC R T H 2をコードする遺伝子の発現方法としては、直接発現以外に、分泌生産又は融合蛋白質発現などの方法 [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press(1989)] を用いることができる。
- [0158] ヒトC R T H 2の生産方法としては、宿主細胞内に生産させる方法、宿主細胞外に分泌させる方法、又は宿主細胞外膜上に生産させる方法があり、使用する宿主細胞、又は生産させるヒトC R T H 2の構造を変えることにより、適切な方法を選択することができる。
- [0159] ヒトC R T H 2が宿主細胞内又は宿主細胞外膜上に生産される場合、ポールソンらの方法 [J. Biol. Chem., 264, 17619(1989)]、ロウらの方法 [Proc. Natl. Acad. Sci. USA, 86, 8227 (1989); Genes Develop., 4, 1288 (199

0)]、日本国特開平05-336963号公報、又は国際公開第94/23021号などに記載の方法を用いることにより、ヒトC R T H 2を宿主細胞外に積極的に分泌させることができる。

[0160] また、ジヒドロ葉酸還元酵素遺伝子などを用いた遺伝子増幅系（日本国特開平02-227075号公報）を利用してヒトC R T H 2の生産量を上昇させることもできる。

[0161] 得られるヒトC R T H 2は、例えば、以下のようにして単離、精製することができる。ヒトC R T H 2が細胞内に溶解状態で発現した場合には、培養終了後に細胞を遠心分離により回収し、水系緩衝液に懸濁後、超音波破砕機、フレンチプレス、マントンガウリンホモゲナイザー、又はダイノミルなどを用いて細胞を破砕し、無細胞抽出液を得る。

[0162] 前記無細胞抽出液を遠心分離することにより得られる上清から、通常の蛋白質の単離精製法、即ち、溶媒抽出法、硫酸などによる塩析法、脱塩法、有機溶媒による沈殿法、ジエチルアミノエチル（D E A E）ーセファロース、D I A I O N H P A - 7 5（三菱化学社）などのレジンを用いた陰イオン交換クロマトグラフィー法、S - S e p h a r o s e F F（ファルマシア社）などのレジンを用いた陽イオン交換クロマトグラフィー法、ブチルセファロース、フェニルセファロースなどのレジンを用いた疎水性クロマトグラフィー法、分子篩を用いたゲルろ過法、アフィニティークロマトグラフィー法、クロマトフォーカシング法、又は等電点電気泳動などの電気泳動法などの手法を単独又は組み合わせて用い、精製標品を得ることができる。

[0163] ヒトC R T H 2が細胞内に不溶体を形成して発現した場合は、上記と同様に細胞を回収後破砕し、遠心分離を行うことにより、沈殿画分として該ヒトC R T H 2の不溶体を回収する。回収した該ヒトC R T H 2の不溶体を蛋白質変性剤で可溶化する。該可溶化液を希釈又は透析することにより、該ヒトC R T H 2を正常な立体構造に戻した後、上記と同様の単離精製法によりポリペプチドの精製標品を得ることができる。

[0164] ヒトC R T H 2又はその糖修飾体などの誘導体が細胞外に分泌された場合

には、培養上清において該ヒトC R T H 2 又はその糖修飾体などの誘導体を回収することができる。該培養物を上記と同様に遠心分離などの手法により処理することにより可溶性画分を取得し、該可溶性画分から、上記と同様の単離精製法を用いることにより、精製標品を得ることができる。

[0165] また、本発明において用いられるヒトC R T H 2 は、F m o c 法、又はt B o c 法などの化学合成法によっても製造することができる。また、アドバンストケムテック社、パーキン・エルマー社、ファルマシア社、プロテインテクノロジーインストルメント社、シンセセルーベガ社、パーセプチブ社、又は島津製作所社などのペプチド合成機を利用して化学合成することもできる。

[0166] (2) 動物の免疫と融合用抗体産生細胞の調製

3～20週令のマウス、ラット又はハムスターなどの動物に、(1)で得られる抗原を免疫して、その動物の脾臓、リンパ節、末梢血中の抗体産生細胞を採取する。また、免疫原性が低く上記の動物で十分な抗体価の上昇が認められない場合には、ヒトC R T H 2 ノックアウトマウスを被免疫動物として用いることもできる。

[0167] 免疫は、動物の皮下、尾根部、静脈内又は腹腔内に、例えば、フロインドの完全アジュバント、又は水酸化アルミニウムゲルと百日咳菌ワクチンなどの適当なアジュバントとともに抗原を投与することにより行う。抗原が部分ペプチドである場合には、B S A (ウシ血清アルブミン)、又はK L H (K e y h o l e L i m p e t H e m o c y a n i n)などのキャリア蛋白質とコンジュゲートを作製し、これを免疫原として用いる。

[0168] 抗原の投与は、1回目の投与の後、1～2週間おきに1～10回行う。各投与後3～7日目に眼底静脈叢あるいは尾静脈より採血し、その血清の抗体価を酵素免疫測定法 [Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)] などを用いて測定する。免疫に用いた抗原に対し、その血清が十分な抗体価を示した動物を融合用抗体産生細胞の供給源とする。

[0169] 抗原の最終投与後3～7日目に、免疫した動物より脾臓などの抗体産生細胞

胞を含む組織を摘出し、抗体産生細胞を採取する。脾臓細胞を用いる場合には、脾臓を細断、ほぐした後、遠心分離し、さらに赤血球を除去して融合用抗体産生細胞を取得する。

[0170] (3) 骨髓腫細胞の調製

骨髓腫細胞としては、マウスから得られる株化細胞を用い、例えば、8-アザグアニン耐性マウス(BALB/c由来)骨髓腫細胞株P3-X63Ag8-U1(P3-U1) [Current Topics in Microbiology and Immunology, 18, 1(1978)]、P3-NS1/1Ag41(NS-1) [European J. Immunology, 6, 511(1976)]、SP2/O-Ag14(SP-2) [Nature, 276, 269(1978)]、P3-X63-Ag8653(653) [J. Immunology, 123, 1548(1979)]、又はP3-X63-Ag8(X63) [Nature, 256, 495(1975)]などを用いる。

[0171] 該骨髓腫細胞は、正常培地[グルタミン、2-メルカプトエタノール、ジェンタマイシン、FBS、及び8-アザグアニンを加えたRPMI 1640培地]で継代し、細胞融合の3~4日前に正常培地に継代し、融合当日 2×10^7 個以上の細胞数を確保する。

[0172] (4) 細胞融合とモノクローナル抗体産生ハイブリドーマの調製

(2)で得られる融合用抗体産生細胞と(3)で得られる骨髓腫細胞をMinimum Essential Medium(MEM)培地又はPBS(リン酸二ナトリウム1.83g、リン酸一カリウム0.21g、食塩7.65g、蒸留水1リットル、pH7.2)でよく洗浄し、細胞数が、融合用抗体産生細胞：骨髓腫細胞=5~10:1になるよう混合し、遠心分離した後、上清を除く。

[0173] 沈澱した細胞群をよくほぐした後、ポリエチレングリコール1000(PEG-1000)、MEM培地及びジメチルスルホキシドの混液を37℃で、攪拌しながら加える。さらに1~2分間毎にMEM培地1~2mLを数回加えた後、MEM培地を加えて全量が50mLになるようにする。遠心分離後、上清を除く。

[0174] 沈澱した細胞群をゆるやかにほぐした後、融合用抗体産生細胞にHAT培地〔ヒポキサンチン、チミジン、及びアミノプテリンを加えた正常培地〕中にゆるやかに細胞を懸濁する。この懸濁液を37℃、5%CO₂インキュベーター中、7～14日間培養する。

[0175] 培養後、培養上清の一部を抜き取り、後述のヒトCRTH2発現細胞に対する反応性解析などのハイブリドーマの選択方法により、ヒトCRTH2を含む抗原に反応し、ヒトCRTH2を含まない抗原に反応しない細胞群を選択する。次に、限界希釈法によりクローニングを2回繰り返し〔1回目はHT培地（HAT培地からアミノプテリンを除いた培地）、2回目は正常培地を使用する〕、安定して強い抗体価の認められるものをモノクローナル抗体産生ハイブリドーマとして選択する。

[0176] (5) 精製モノクローナル抗体の調製

プリスタン処理〔2, 6, 10, 14-テトラメチルペンタデカン (Pristan) 0.5 mLを腹腔内投与し、2週間飼育する〕した8～10週令のマウス又はヌードマウスに、(4)で得られるモノクローナル抗体産生ハイブリドーマを腹腔内に注射する。10～21日でハイブリドーマは腹水がん化する。このマウスから腹水を採取し、遠心分離して固形分を除去後、40～50%硫酸アンモニウムで塩析し、カプリル酸沈殿法、DEAEセファロースカラム、プロテインAカラム又はゲル濾過カラムによる精製を行ない、IgG又はIgM画分を集め、精製モノクローナル抗体とする。

[0177] また、(4)で得られるモノクローナル抗体産生ハイブリドーマを、10%FBS添加を添加したRPMI 1640培地などで培養した後、遠心分離により上清を除き、Hybridoma-SFM培地に懸濁し、3～7日間培養する。得られる細胞懸濁液を遠心分離し、得られる上清よりプロテインAカラム又はプロテインGカラムによる精製を行ない、IgG画分を集め、精製モノクローナル抗体を得ることもできる。なお、Hybridoma-SFM培地には5%ダイゴGF21を添加することもできる。

[0178] 抗体のサブクラスの決定は、サブクラスタイピングキットを用いて酵素免

疫測定法により行う。蛋白量の定量は、ローリー法又は280nmでの吸光度より算出する。

[0179] (6) モノクローナル抗体の選択

モノクローナル抗体の選択は、以下に示す通り、ヒトC R T H 2を発現する細胞に対する反応性をフローサイトメトリーで解析することにより行う。

[0180] ヒトC R T H 2を発現する細胞としては、例えば(1)で得られるヒトC R T H 2をコードするc D N Aを含む発現ベクターを動物細胞などに導入して得られる遺伝子導入細胞や、ヒトの好酸球、好塩基球、T h 2、I L C 2、n o n c l a s s i c a l m o n o c y t eおよびT h 2 / T h 1 7細胞などが挙げられる。

[0181] 細胞を96ウェルプレートなどのプレートに分注した後、第1抗体として血清、ハイブリドーマの培養上清又は精製モノクローナル抗体などの被験物質を分注し、反応させる。反応後の細胞を1~10% b o v i n e s e r u m a l b u m i n (B S A) を含むP B S (以下、B S A - P B Sと記す)などで、よく洗浄した後、第2抗体として蛍光試薬などで標識した抗イムノグロブリン抗体を分注して反応させる。B S A - P B Sなどでよく洗浄した後、フローサイトメーターを用いて標識化抗体の蛍光量を測定することにより、発現細胞に対して特異的に反応するモノクローナル抗体を選択する。

[0182] また、本発明のモノクローナル抗体と競合してヒトC R T H 2に結合するモノクローナル抗体は、上述のフローサイトメトリーを用いた結合反応検出系に、被検抗体を添加して反応させることで取得できる。

[0183] すなわち、被検抗体を加えた時に本発明のモノクローナル抗体の結合が阻害される抗体をスクリーニングすることにより、ヒトC R T H 2のアミノ酸配列、又はその立体構造への結合について、本発明で取得したモノクローナル抗体と競合するモノクローナル抗体を取得することができる。

[0184] さらに、本発明のヒトC R T H 2のアミノ酸配列、又はその立体構造に結合するモノクローナル抗体が認識するエピトープと同じエピトープに結合す

る抗体は、上述のフローサイトメトリーを用いた結合反応検出系で取得された抗体のエピトープを同定し、同定したエピトープの、部分的な合成ペプチド、又はエピトープの立体構造に擬態させた合成ペプチド等を作製し、免疫することで、取得することができる。

[0185] 2. 遺伝子組換え抗体の作製

遺伝子組換え抗体の作製例として、以下にヒト型キメラ抗体及びヒト化抗体の作製方法を示す。

(1) 遺伝子組換え抗体発現用ベクターの構築

遺伝子組換え抗体発現用ベクターは、ヒト抗体のC H及びC LをコードするDNAが組み込まれた動物細胞用発現ベクターであり、動物細胞用発現ベクターにヒト抗体のC H及びC LをコードするDNAをそれぞれクローニングすることにより構築することができる。

[0186] ヒト抗体のC領域は任意のヒト抗体のC H及びC Lを用いることができる。例えば、ヒト抗体の γ 1サブクラスのC H及び κ クラスのC Lなどを用いる。ヒト抗体のC H及びC LをコードするDNAには、cDNAを用いるが、エキソンとイントロンからなる染色体DNAを用いることもできる。

[0187] 動物細胞用発現ベクターには、ヒト抗体のC領域をコードする遺伝子を組み込み発現できるものであればいかなるものでも用いることができる。例えば、pAGE107 [Cytotechnol., 3, 133(1990)]、pAGE103 [J. Biochem., 101, 1307(1987)]、pHSG274 [Gene, 27, 223(1984)]、pKCR [Proc. Natl. Acad. Sci. USA, 78, 1527(1981)]、pSG1bd2-4 [Cytotechnol., 4, 173(1990)]、又はpSE1UK1Seed1-3 [Cytotechnol., 13, 79(1993)]などを用いる。

[0188] 動物細胞用発現ベクターのうちプロモーターとエンハンサーには、SV40の初期プロモーター [J. Biochem., 101, 1307(1987)]、モロニーマウス白血病ウイルスLTR [Biochem. Biophys. Res. Commun., 149, 960(1987)]、又は免疫グロブリンH鎖のプロモーター [Cell, 41, 479(1985)]とエンハンサー [Cell, 33, 717(1983)]などを用いる。

[0189] 遺伝子組換え抗体発現用ベクターには、遺伝子組換え抗体発現ベクターの構築の容易さ、動物細胞への導入の容易さ、動物細胞内での抗体H鎖及びL鎖の発現量のバランスが均衡するなどの点から、抗体H鎖及びL鎖が同一のベクター上に存在するタイプ(タンデム型)の遺伝子組換え抗体発現用ベクター [J. Immunol. Methods, 167, 271 (1994)] を用いるが、抗体H鎖及びL鎖が別々のベクター上に存在するセパレートベクターを用いることもできる。タンデム型の遺伝子組換え抗体発現用ベクターには、pKANTE X 93 (国際公開第97/10354号)、pEE18 [Hybridoma, 17, 559 (1998)] などを用いる。

[0190] (2) ヒト以外の動物由来の抗体のV領域をコードするcDNAの取得及びアミノ酸配列の解析

非ヒト抗体のVH及びVLをコードするcDNAの取得及びアミノ酸配列の解析は以下のようにして行うことができる。

[0191] 非ヒト抗体を産生するハイブリドーマ細胞よりmRNAを抽出し、cDNAを合成する。合成したcDNAをファージ又はプラスミドなどのベクターにクローニングしてcDNAライブラリーを作製する。

[0192] 前記ライブラリーより、マウス抗体のC領域部分又はV領域部分をコードするDNAをプローブとして用い、VH又はVLをコードするcDNAを有する組換えファージ又は組換えプラスミドをそれぞれ単離する。組換えファージ又は組換えプラスミド上の目的とするマウス抗体のVH又はVLの全塩基配列をそれぞれ決定し、塩基配列よりVH又はVLの全アミノ酸配列をそれぞれ推定する。

[0193] 非ヒト抗体を産生するハイブリドーマ細胞を作製するヒト以外の動物には、マウス、ラット、ハムスター、又はラビットなどを用いるが、ハイブリドーマ細胞を作製することが可能であれば、いかなる動物も用いることができる。

[0194] ハイブリドーマ細胞からの全RNAの調製には、チオシアン酸グアニジントリフルオロ酢酸セシウム法 [Methods in Enzymol., 154, 3 (1987)]、

又はRNA easy kit (キアゲン社) などのキットなどを用いる。

[0195] 全RNAからのmRNAの調製には、オリゴ(dT)固定化セルロースカラム法 [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)]、又はOligo-dT30<Super>mRNA Purification Kit (タカラバイオ社) などのキットなどを用いる。また、Fast Track mRNA Isolation Kit (インビトロジェン社)、又はQuickPrep mRNA Purification Kit (ファルマシア社) などのキットを用いてハイブリドーマ細胞からmRNAを調製することもできる。

[0196] cDNAの合成及びcDNAライブラリーの作製には、公知の方法 [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press(1989), Current Protocols in molecular Biology, Supplement 1, John Wiley&Sons(1987-1997)]、SperScript Plasmid System for cDNA Synthesis and Plasmid Cloning(インビトロジェン社)、又はZAP-cDNA Synthesis Kit (ストラタジーン社) などのキットなどを用いる。

[0197] cDNAライブラリーの作製の際、ハイブリドーマ細胞から抽出したmRNAを鋳型として合成したcDNAを組み込むベクターには、該cDNAを組み込めるベクターであればいかなるものでも用いることができる。

[0198] 例えば、ZAP Express [Strategies, 5, 58(1992)]、pBlue-script II SK (+) [Nucleic Acids Research, 17, 9494(1989)]、λZAP II (ストラタジーン社)、λgt10、λgt11 [DNA Cloning: A Practical Approach, I, 49(1985)]、Lambda BlueMid (クローンテック社)、λEx Cell、pT7T3-18U (ファルマシア社)、pcD2 [Mol. Cell. Biol., 3, 280 (1983)]、又はpUC18 [Gene, 33, 103 (1985)] などを用いる。

- [0199] ファージ又はプラスミドベクターにより構築されるcDNAライブラリーを導入する大腸菌には、該cDNAライブラリーを導入、発現及び維持できるものであればいかなるものでも用いることができる。例えば、XL-1 Blue MRF [Strategies, 5, 81(1992)]、C600 [Genetics, 39, 440(1954)]、Y1088、Y1090 [Science, 222, 778(1983)]、NM522 [J. Mol. Biol., 166, 1(1983)]、K802 [J. Mol. Biol., 16, 118(1966)]、又はJM105 [Gene, 38, 275(1985)]などを用いる。
- [0200] cDNAライブラリーからの非ヒト抗体のVH又はVLをコードするcDNAクローンの選択には、アイソトープ又は蛍光標識したプローブを用いたコロニー・ハイブリダイゼーション法、又はプラーク・ハイブリダイゼーション法 [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press(1989)]などを用いる。
- [0201] また、プライマーを調製し、mRNAから合成したcDNA又はcDNAライブラリーを鋳型として、Polymerase Chain Reaction法 [以下、PCR法と記す、Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press(1989), Current Protocols in molecular Biology, Supplement 1, John Wiley & Sons(1987-1997)]を行うことよりVH又はVLをコードするcDNAを調製することもできる。
- [0202] 選択されたcDNAを、適当な制限酵素などで切断後、pBlue-script SK(−) (ストラタジーン社)などのプラスミドにクローニングし、通常用いられる塩基配列解析方法などにより該cDNAの塩基配列を決定する。塩基配列解析方法には、例えば、ジデオキシ法 [Proc. Natl. Acad. Sci. USA, 74, 5463(1977)]などの反応を行った後、ABI PRISM 3700 (PEバイオシステムズ社)又はA. L. F. DNAシーケンサー (ファルマシア社)などの塩基配列自動分析装置などを用いる。
- [0203] 決定した塩基配列からVH及びVLの全アミノ酸配列をそれぞれ推定し、既知の抗体のVH及びVLの全アミノ酸配列 [A. L. F. DNA, US Dept. Health

and Human Services(1991)] と比較することにより、取得した cDNA が分泌シグナル配列を含む抗体の V_H 及び V_L の完全なアミノ酸配列をコードしているかをそれぞれ確認する。

[0204] 分泌シグナル配列を含む抗体の V_H 及び V_L の完全なアミノ酸配列に関しては、既知の抗体の V_H 及び V_L の全アミノ酸配列 [A.L.F.DNA, US Dept. Health and Human Services(1991)] と比較することにより、分泌シグナル配列の長さ及び N 末端アミノ酸配列を推定でき、さらにはそれらが属するサブグループを知ることができる。また、V_H 及び V_L の各 CDR のアミノ酸配列についても、既知の抗体の V_H 及び V_L のアミノ酸配列 [A.L.F.DNA, US Dept. Health and Human Services(1991)] と比較することによって見出すことができる。

[0205] また、得られる V_H 及び V_L の完全なアミノ酸配列を用いて、例えば、SWISS-PROT 又は PIR-Protein などの任意のデータベースに対して BLAST 法 [J. Mol. Biol., 215, 403(1990)] などの相同性検索を行い、V_H 及び V_L の完全なアミノ酸配列が新規なものかを確認できる。

[0206] (3) ヒト型キメラ抗体発現ベクターの構築

(1) で得られる遺伝子組換え抗体発現用ベクターのヒト抗体の C_H 又は C_L をコードするそれぞれの遺伝子の上流に、それぞれ非ヒト抗体の V_H 又は V_L をコードする cDNA をそれぞれクローニングすることで、ヒト型キメラ抗体発現ベクターを構築することができる。

[0207] 非ヒト抗体の V_H 又は V_L をコードする cDNA の 3' 末端側と、ヒト抗体の C_H 又は C_L の 5' 末端側とを連結するために、連結部分の塩基配列が適切なアミノ酸をコードし、かつ適当な制限酵素認識配列になるように設計した V_H 及び V_L の cDNA を作製する。

[0208] 作製された V_H 及び V_L の cDNA を、(1) で得られるヒト化抗体発現用ベクターのヒト抗体の C_H 又は C_L をコードするそれぞれの遺伝子の上流にそれらが適切な形で発現する様にそれぞれクローニングし、ヒト型キメラ抗体発現ベクターを構築する。

[0209] また、非ヒト抗体V_H又はV_LをコードするcDNAを、適当な制限酵素の認識配列を両端に有する合成DNAを用いてPCR法によりそれぞれ増幅し、(1)で得られる遺伝子組換え抗体発現用ベクターにクローニングすることもできる。

[0210] (4) ヒト化抗体のV領域をコードするcDNAの構築

ヒト化抗体のV_H又はV_LをコードするcDNAは、以下のようにして構築することができる。

[0211] 非ヒト抗体のV_H又はV_LのCDRのアミノ酸配列を移植するヒト抗体のV_H又はV_LのFRのアミノ酸配列をそれぞれ選択する。選択するFRのアミノ酸配列には、ヒト抗体由来のものであれば、いずれのものでも用いることができる。

[0212] 例えば、Protein Data Bankなどのデータベースに登録されているヒト抗体のFRのアミノ酸配列、又はヒト抗体のFRの各サブグループの共通アミノ酸配列[A.L.F.DNA, US Dept. Health and Human Services(1991)]などを用いる。抗体の結合活性の低下を抑えるため、元の抗体のV_H又はV_LのFRのアミノ酸配列とできるだけ高い相同性(少なくとも60%以上)のFRのアミノ酸配列を選択する。

[0213] 次に、選択したヒト抗体のV_H又はV_LのFRのアミノ酸配列に、もとの抗体のCDRのアミノ酸配列をそれぞれ移植し、ヒト化抗体のV_H又はV_Lのアミノ酸配列をそれぞれ設計する。設計したアミノ酸配列を抗体の遺伝子の塩基配列に見られるコドンの使用頻度[A.L.F.DNA, US Dept. Health and Human Services(1991)]を考慮してDNA配列に変換し、ヒト化抗体のV_H又はV_Lのアミノ酸配列をコードするDNA配列をそれぞれ設計する。

[0214] 設計したDNA配列に基づき、100塩基前後の長さからなる数本の合成DNAを合成し、それらを用いてPCR反応を行う。この場合、PCR反応での反応効率及び合成可能なDNAの長さから、好ましくはH鎖、L鎖それぞれについて各6本の合成DNAを設計する。また、両端に位置する合成DNAの5'末端に適当な制限酵素の認識配列を導入することで、容易に、ヒ

ト化抗体のVH又はVLをコードするcDNAを、(1)で得られるヒト化抗体発現用ベクターに、クローニングすることができる。

[0215] PCR反応後、増幅産物をpBluescript SK(−)(ストラタジーン社)などのプラスミドにそれぞれクローニングし、(2)に記載の方法と同様の方法により、塩基配列を決定し、所望のヒト化抗体のH鎖全長又はL鎖全長のアミノ酸配列をコードするDNA配列を有するプラスミドを取得する。

[0216] または、設計したDNA配列に基づき、VH全長およびVL全長を各々1本の長鎖DNAとして合成したものを、上記PCR増幅産物に代えて用いることもできる。さらに、合成長鎖DNAの両端に適当な制限酵素の認識配列を導入することで、ヒト化抗体のVHまたはVLをコードするcDNAを、(1)で得られるヒト化抗体発現用ベクターへ容易にクローニングすることができる。

[0217] (5) ヒト化抗体のV領域のアミノ酸配列の改変

ヒト化抗体は、非ヒト抗体のVH及びVLのCDRのみをヒト抗体のVH及びVLのFRに移植しただけでは、その抗原結合活性は元の非ヒト抗体に比べて低下する[BIO/TECHNOLOGY, 9, 266 (1991)]。ヒト化抗体では、ヒト抗体のVH及びVLのFRのアミノ酸配列の中で、直接抗原との結合に参与しているアミノ酸残基、CDRのアミノ酸残基と相互作用するアミノ酸残基、及び抗体の立体構造を維持し、間接的に抗原との結合に参与しているアミノ酸残基を同定し、それらのアミノ酸残基を元の非ヒト抗体のアミノ酸残基に置換することにより、低下した抗原結合活性を上昇させることができる。

[0218] 抗原結合活性に関わるFRのアミノ酸残基を同定するために、X線結晶解析[J. Mol. Biol., 112, 535 (1977)]又はコンピューターモデリング[Protein Engineering, 7, 1501 (1994)]などを用いることにより、抗体の立体構造の構築及び解析を行うことができる。また、それぞれの抗体について数種の改変体を作製し、それぞれの抗原結合活性との相関を検討することを繰り返し、試行錯誤することで必要な抗原結合活性を有するヒト化抗体を取得

できる。

[0219] ヒト抗体のVH及びVLのFRのアミノ酸残基は、改変用合成DNAを用いて(4)に記載のPCR反応を行うことにより、置換することができる。PCR反応後の増幅産物について(2)に記載の方法により、塩基配列を決定し、目的の改変が施されたことを確認する。

[0220] (6) ヒト化抗体発現ベクターの構築

(1) で得られる遺伝子組換え抗体発現用ベクターのヒト抗体のCH又はCLをコードするそれぞれの遺伝子の上流に、構築した遺伝子組換え抗体のVH又はVLをコードするcDNAをそれぞれクローニングし、ヒト化抗体発現ベクターを構築することができる。

[0221] 例えば、(4) 及び(5) で得られるヒト化抗体のVH又はVLを構築する際に用いる合成DNAのうち、両端に位置する合成DNAの5' 末端に適当な制限酵素の認識配列を導入することで、(1) で得られるヒト化抗体発現用ベクターのヒト抗体のCH又はCLをコードするそれぞれの遺伝子の上流にそれらが適切な形で発現するようにそれぞれクローニングする。

[0222] (7) 遺伝子組換え抗体の一過性発現

(3) 及び(6) で得られる遺伝子組換え抗体発現ベクター、又はそれらを改変した発現ベクターを用いて遺伝子組換え抗体の一過性発現を行い、作製した多種類の遺伝子組換え抗体の抗原結合活性を効率的に評価することができる。

[0223] 発現ベクターを導入する宿主細胞には、遺伝子組換え抗体を発現できる宿主細胞であれば、いかなる細胞でも用いることができるが、例えばCOS-7細胞(ATCC番号:CRL1651)を用いる[Methods in Nucleic Acids Res., CRC Press, 283(1991)]。

COS-7細胞への発現ベクターの導入には、DEAE-デキストラン法[Methods in Nucleic Acids Res., CRC Press(1991)]、又はリポフェクション法[Proc. Natl. Acad. Sci. USA, 84, 7413(1987)]などを用いる。

[0224] 発現ベクターの導入後、培養上清中の遺伝子組換え抗体の発現量及び抗原

結合活性は酵素免疫抗体法 [Monoclonal Antibodies-Principles and practice, Third edition, Academic Press(1996); Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory(1988)、単クローン抗体実験マニュアル、講談社サイエンティフィック(1987)] などを用いて測定する。

[0225] (8) 遺伝子組換え抗体を安定に発現する形質転換株の取得と遺伝子組換え抗体の調製

(3) 及び (6) で得られる遺伝子組換え抗体発現ベクターを適当な宿主細胞に導入することにより遺伝子組換え抗体を安定に発現する形質転換株を得ることができる。

[0226] 宿主細胞への発現ベクターの導入には、エレクトロポレーション法 [日本特開平02-257891号公報; Cytotechnology, 3, 133(1990)] などを用いる。

[0227] 遺伝子組換え抗体発現ベクターを導入する宿主細胞には、遺伝子組換え抗体を発現させることができる宿主細胞であれば、いかなる細胞でも用いることができる。例えば、CHO-K1 (ATCC番号: CCL-61)、DukXB11 (ATCC番号: CCL-9096)、Pro-5 (ATCC番号: CCL-1781)、CHO-S (Life Technologies, Cat # 11619)、ラットミエローマ細胞 YB2/3HL.P2.G11.16Ag.20 (又は YB2/0ともいう)、マウスミエローマ細胞 NS0、マウスミエローマ細胞 SP2/0-Ag14 (ATCC番号: CRL1581)、マウス P3-X63-Ag8653細胞 (ATCC番号: CRL1580)、ジヒドロ葉酸還元酵素遺伝子 (Dihydrofolate Reductase、以下、DHFRと表記する) が欠損した CHO細胞 [Proc. Natl. Acad. Sci. USA, 77, 4216(1980)]、レクチン耐性を獲得した Lec13 [Somatic Cell and Molecular Genetics, 12, 55(1986)]、 α 1,6-フコース転移酵素遺伝子が欠損した CHO細胞 (国際公開第2005/035586号、国際公開第02/31140号)、ラット YB2/3HL.P2.G11.16Ag.20細胞 (ATCC番号: CRL1662

）などを用いる。

[0228] 発現ベクターの導入後、遺伝子組換え抗体を安定に発現する形質転換株は、G 4 1 8 硫酸塩などの薬剤を含む動物細胞培養用培地で培養することにより選択する（日本国特開平 0 2 - 2 5 7 8 9 1 号公報）。

[0229] 動物細胞培養用培地には、R P M I 1 6 4 0 培地（インビトロジェン社）、G I T 培地（日本製薬社）、E X - C E L L 3 0 1 培地（ジェイアールエイチ社）、I M D M 培地（インビトロジェン社）、H y b r i d o m a - S F M 培地（インビトロジェン社）、又はこれら培地に F B S などの各種添加物を添加した培地などを用いる。

[0230] 得られる形質転換株を培地中で培養することで培養上清中に遺伝子組換え抗体を発現蓄積させる。培養上清中の遺伝子組換え抗体の発現量及び抗原結合活性は E L I S A 法などにより測定できる。また、D H F R 増幅系（日本国特開平 0 2 - 2 5 7 8 9 1 号公報）などを利用して、形質転換株が産生する遺伝子組換え抗体の発現量を上昇させることができる。

[0231] 遺伝子組換え抗体は、形質転換株の培養上清よりプロテイン A - カラムを用いて精製する [Monoclonal Antibodies-Principles and practice, Third edition, Academic Press (1996); Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)]。また、ゲル濾過、イオン交換クロマトグラフィー及び限外濾過などの蛋白質の精製で用いられる方法を組み合わせることもできる。

[0232] 精製した遺伝子組換え抗体の H 鎖、L 鎖または抗体分子全体の分子量は、ポリアクリルアミドゲル電気泳動法 [Nature, 227, 680(1970)]、又はウェスタンブロッティング法 [Monoclonal Antibodies-Principles and practice, Third edition, Academic Press(1996); Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory(1988)] など用いて測定することができる。

[0233] 3. 精製モノクローナル抗体又は該抗体断片の活性評価

精製した本発明のモノクローナル抗体又は該抗体断片の活性評価は、以下

のように行うことができる。

[0234] ヒトC R T H 2 発現細胞株に対する結合活性は、例えば前述の1. (6) 記載のフローサイトメトリーを用いた結合反応検出系などの蛍光抗体法 [Cancer Immunol. Immunother., 36, 373(1993)] を用いて測定できる。

[0235] ヒトC R T H 2 陽性培養細胞株に対するC D C 活性、又はA D C C 活性は公知の測定方法 [Cancer Immunol. Immunother., 36, 373(1993); Current protocols in Immunology, Chapter7. Immunologic studies in humans, Editor, John E, Coligan et al., John Wiley & Sons, Inc., (1993)] により測定する。

[0236] 4. 抗体のエフェクター活性を制御する方法

本発明のモノクローナル抗体のエフェクター活性を制御する方法としては、抗体のF c 領域の297番目のアスパラギン (A s n) に結合するN結合複合型糖鎖の還元末端に存在するN-アセチルグルコサミン (G l c N A c) に α 1, 6結合するフコース (コアフコースともいう) の量を制御する方法 (国際公開第2005/035586号、国際公開第2002/31140号、国際公開第00/61739号)、又は抗体のF c 領域のアミノ酸残基を改変することで制御する方法などが知られている。本発明のモノクローナル抗体にはいずれの方法を用いても、エフェクター活性を制御することができる。

[0237] エフェクター活性とは、抗体のF c 領域を介して引き起こされる抗体依存性の活性をいい、A D C C 活性、C D C 活性、又はマクロファージ若しくは樹状細胞などの食細胞による抗体依存性ファゴサイトーシス (A n t i b o d y - d e p e n d e n t p h a g o c y t o s i s、A D P 活性) などが知られている。

[0238] エフェクター活性の測定法として、例えば、標的として炎症性細胞、エフェクターとしてヒト末梢血単核球 (P B M C)、そして炎症性細胞特異的な抗体を混合し、4時間程度インキュベーションした後、細胞障害の指標として遊離してきた乳酸脱水素酵素 (L D H) を測定することができる。もしくは

は、ヒト P B M C に、例えば C D 2 0 の様な血液細胞特異的な抗原を認識する抗体を混合し、インキュベーションした後、遊離 L D H の測定やフローサイトメトリーによる該細胞数の減少をエフェクター活性として測定することができる。

[0239] 抗体の F c の N 結合複合型糖鎖のコアフコースの含量を制御することで、抗体のエフェクター活性を増加又は低下させることができる。抗体の F c に結合している N 結合複合型糖鎖に結合するフコースの含量を低下させる方法としては、例えば、 $\alpha 1, 6$ -フコース転移酵素遺伝子が欠損した C H O 細胞を用いて抗体を発現させる方法が挙げられ、フコースが結合していない抗体を取得することができる。フコースが結合していない抗体は高い A D C C 活性を有する。

[0240] 一方、抗体の F c に結合している N 結合複合型糖鎖に結合するフコースの含量を増加させる方法としては、例えば、 $\alpha 1, 6$ -フコース転移酵素遺伝子を導入した宿主細胞を用いて抗体を発現させる方法が挙げられ、フコースが結合している抗体を取得することができる。フコースが結合している抗体は、フコースが結合していない抗体よりも低い A D C C 活性を有する。

[0241] また、抗体の F c 領域のアミノ酸残基を改変することで A D C C 活性又は C D C 活性を増加又は低下させることができる。例えば、米国特許公開第 2 0 0 7 / 0 1 4 8 1 6 5 号明細書に記載の F c 領域のアミノ酸配列を用いることで、抗体の C D C 活性を増加させることができる。

[0242] また、米国特許第 6 7 3 7 0 5 6 号明細書、米国特許第 7 2 9 7 7 7 5 号明細書又は米国特許第 7 3 1 7 0 9 1 号明細書に記載のアミノ酸改変を行うことで、A D C C 活性又は C D C 活性を、増加させることも低下させることもできる。

[0243] また本発明の抗体には、上述の抗体定常領域におけるアミノ酸改変や糖鎖改変に合わせて例えば日本国特開 2 0 1 3 - 1 6 5 7 1 6 号公報、日本国特開 2 0 1 2 - 0 2 1 0 0 4 号公報などに記載のアミノ酸改変を行うことにより、F c 受容体への反応性を制御することで血中半減期を制御した抗体も含

まれる。

[0244] さらに、上述の方法を組み合わせ、一つの抗体に使用することにより、抗体のエフェクター活性や血中半減期が制御された抗体を取得することができる。

[0245] 5. 本発明のモノクローナル抗体又は該抗体断片を用いた疾患の治療方法

本発明の抗体は、ヒトC R T H 2 が関係する疾患の治療に用いることができる。投与経路としては、例えば、経口投与、又は口腔内、気道内、直腸内、皮下、筋肉内、静脈内、若しくは腹腔内などの非経口投与が挙げられる。投与形態としては、例えば、噴霧剤、カプセル剤、錠剤、散剤、顆粒剤、シロップ剤、乳剤、坐剤、注射剤、軟膏、又はテープ剤などが挙げられる。

[0246] 経口投与に適当な製剤としては、例えば、乳剤、シロップ剤、カプセル剤、錠剤、散剤、又は顆粒剤などが挙げられる。

乳剤又はシロップ剤のような液体調製物は、水、ショ糖、ソルビトール若しくは果糖などの糖類、ポリエチレングリコール若しくはプロピレングリコールなどのグリコール類、ごま油、オリーブ油若しくは大豆油などの油類、p-ヒドロキシ安息香酸エステル類などの防腐剤、又はストロベリーフレーバー若しくはペパーミントなどのフレーバー類などを添加剤として用いて製造する。

[0247] カプセル剤、錠剤、散剤又は顆粒剤などは、乳糖、ブドウ糖、ショ糖若しくはマンニトールなどの賦形剤、デンプン若しくはアルギン酸ナトリウムなどの崩壊剤、ステアリン酸マグネシウム若しくはタルクなどの滑沢剤、ポリビニルアルコール、ヒドロキシプロピルセルロース若しくはゼラチンなどの結合剤、脂肪酸エステルなどの界面活性剤又はグリセリンなどの可塑剤などを添加剤として用いて製造する。

[0248] 非経口投与に適当な製剤としては、例えば、注射剤、坐剤、又は噴霧剤などが挙げられる。

[0249] 注射剤は、塩溶液、ブドウ糖溶液、又はその両者の混合物からなる担体などを用いて製造する。

[0250] 坐剤はカカオ脂、水素化脂肪、又はカルボン酸などの担体を用いて製造する。

[0251] 噴霧剤は受容者の口腔及び気道粘膜を刺激せず、かつ本発明のモノクローナル抗体又は該抗体断片を微細な粒子として分散させ、吸収を容易にさせる担体などを用いて製造する。担体としては、例えば乳糖又はグリセリンなどを用いる。また、エアロゾル又はドライパウダーとして製造することもできる。

[0252] さらに、上記非経口剤においても、経口投与に適当な製剤で添加剤として例示した成分を添加することもできる。

[0253] 6. 本発明の抗体又は該抗体断片を用いた疾患の診断方法

本発明の抗体又は該抗体断片を用いて、ヒトC R T H 2又はヒトC R T H 2が発現した細胞を検出又は測定することにより、ヒトC R T H 2に関連する疾患を診断することができる。

[0254] ヒトC R T H 2に関連する疾患の一つであるアレルギー疾患の診断は、例えば、患者由来の末梢血、喀痰、鼻汁または肺胞洗浄液などに存在する炎症性細胞に発現しているヒトC R T H 2をフローサイトメーターなどの免疫学的手法を用いて検出することにより行うことができる。

[0255] 免疫学的手法とは、標識を施した抗原又は抗体を用いて、抗体量又は抗原量を検出又は測定する方法である。例えば、放射性物質標識免疫抗体法、酵素免疫測定法、蛍光免疫測定法、発光免疫測定法、ウエスタンブロット法又は物理化学的手法などを用いる。

[0256] 放射性物質標識免疫抗体法は、例えば、抗原又は抗原を発現した細胞などに、本発明の抗体又は該抗体断片を反応させ、さらに放射線標識を施した抗イムノグロブリン抗体又は結合断片を反応させた後、シンチレーションカウンタなどで測定する。

[0257] 酵素免疫測定法は、例えば、抗原又は抗原を発現した細胞などに、本発明の抗体又は該抗体断片を反応させ、さらに標識を施した抗イムノグロブリン抗体又は結合断片を反応させた後、発色色素を吸光光度計で測定する。例え

ばサンドイッチELISA法などを用いる。

[0258] 酵素免疫測定法で用いる標識体としては、公知〔酵素免疫測定法、医学書院（1987）〕の酵素標識を用いることができる。例えば、アルカリフォスファターゼ標識、ペルオキシダーゼ標識、ルシフェラーゼ標識、又はビオチン標識などを用いる。

[0259] サンドイッチELISA法は、固相に抗体を結合させた後、検出又は測定対象である抗原をトラップさせ、トラップされた抗原に第2の抗体を反応させる方法である。該ELISA法では、検出又は測定したい抗原を認識する抗体又は抗体断片であって、抗原認識部位の異なる2種類の抗体を準備し、そのうち、第1の抗体又は抗体断片を予めプレート（例えば、96ウェルプレート）に吸着させ、次に第2の抗体又は抗体断片をFITCなどの蛍光物質、ペルオキシダーゼなどの酵素、又はビオチンなどで標識しておく。

[0260] 上記の抗体が吸着したプレートに、生体内から分離された、細胞又はその破碎液、組織又はその破碎液、細胞培養上清、血清、胸水、腹水、又は眼液などを反応させた後、標識したモノクローナル抗体又は抗体断片を反応させ、標識物質に応じた検出反応を行う。濃度既知の抗原を段階的に希釈して作製した検量線より、被験サンプル中の抗原濃度を算出する。

[0261] サンドイッチELISA法に用いる抗体としては、ポリクローナル抗体又はモノクローナル抗体のいずれを用いてもよく、Fab、Fab'、又はF(ab')₂などの抗体フラグメントを用いてもよい。サンドイッチELISA法で用いる2種類の抗体の組み合わせとしては、異なるエピトープを認識するモノクローナル抗体又は抗体断片の組み合わせでもよいし、ポリクローナル抗体とモノクローナル抗体又は抗体断片との組み合わせでもよい。

[0262] 蛍光免疫測定法は、文献〔Monoclonal Antibodies-Principles and practice, Third edition, Academic Press (1996)、単クローン抗体実験マニュアル、講談社サイエンティフィック(1987)〕などに記載された方法で測定する。蛍光免疫測定法で用いる標識体としては、例えば、公知〔蛍光抗体法、ソフトサイエンス社（1983）〕の蛍光標識が挙げられる。例えば、フルオ

レセイニンイソチオシアネート（FITC）、又はテトラメチルローダミンイソチオシアネート（RITC）などが挙げられる。

[0263] 発光免疫測定法は文献〔生物発光と化学発光 臨床検査42、廣川書店（1998）〕などに記載された方法で測定する。発光免疫測定法で用いる標識体としては、例えば、公知の発光体標識が挙げられ、アクリジニウムエステル、ロフィンなどが挙げられる。

[0264] ウェスタンブロット法は、抗原又は抗原を発現した細胞などをSDS（デシル硫酸ナトリウム）-PAGE〔Antibodies-A Laboratory Manual Cold Spring Harbor Laboratory (1988)〕で分画した後、該ゲルをポリフッ化ビニリデン（PVDF）膜又はニトロセルロース膜にブロッティングし、該膜に抗原を認識する抗体又は抗体断片を反応させ、さらにFITCなどの蛍光物質、ペルオキシダーゼなどの酵素標識、又はビオチン標識などを施した抗マウスIgG抗体又は結合断片を反応させた後、該標識を可視化することによって測定する。一例を以下に示す。

[0265] 配列番号2で表されるアミノ酸配列を有するポリペプチドを発現している細胞又は組織を溶解し、還元条件下でレーンあたりの蛋白量として0.1～30μgをSDS-PAGE法により泳動する。泳動された蛋白質をPVDF膜にトランスファーし1～10%BSA-PBSに室温で30分間反応させブロッキング操作を行う。

[0266] ここで本発明の抗体を反応させ、0.05～0.1%のTween-20を含むPBS（以下、Tween-PBSと記す）で洗浄し、ペルオキシダーゼ標識したヤギ抗マウスIgGを室温で2時間反応させる。Tween-PBSで洗浄し、ECL Western Blotting Detection Reagents（アマシャム社製）などを用いてモノクローナル抗体が結合したバンドを検出することにより、配列番号2で表されるアミノ酸配列を有するポリペプチドを検出する。ウェスタンブロッティングでの検出に用いられる抗体としては、天然型の立体構造を保持していないポリペプチドに結合できる抗体が用いられる。

[0267] 物理化学的手法は、例えば、抗原であるヒトC R T H 2と本発明のモノクローナル抗体又は該抗体断片とを結合させることにより凝集体を形成させて、この凝集体を検出することにより行う。この他に物理化学的手法として、例えば、毛細管法、一次元免疫拡散法、免疫比濁法又はラテックス免疫比濁法〔臨床検査法提要、金原出版（1998）〕などが挙げられる。

[0268] ラテックス免疫比濁法は、抗体又は抗原を感作させた粒径0.1～1 μ m程度のポリスチレンラテックスなどの担体を用い、対応する抗原又は抗体により抗原抗体反応を起こさせると、反応液中の散乱光は増加し、透過光は減少する。この変化を吸光度又は積分球濁度として検出することにより被験サンプル中の抗原濃度などを測定する。

[0269] 本発明の抗体又は該抗体断片を用いて、当該抗体による治療有効性を治療開始前に判断する方法としては、例えば以下が挙げられる。

[0270] まず、治療開始前に、患者体内から例えば末梢血、喀痰、肺胞洗浄液、鼻汁などを採取し、その懸濁液に本発明の抗体又は該抗体断片を添加し、一定時間後、炎症性細胞の除去やT h 2タイプサイトカインなどの生体機能分子に対する阻害活性をはじめとした、抗炎症性細胞活性を測定する。測定の結果、抗炎症性細胞活性が検出された場合、その末梢血、喀痰、鼻汁などを有する患者の治療には、本発明の抗体又は該抗体断片が有効であると治療開始前に判断することができる。

実施例

[0271] 以下に、実施例により本発明を説明するが、本発明はこれらに限定されるものではない。

[0272] [実施例1]

ヒトC R T H 2発現細胞の造成

(1) ヒトC R T H 2発現ベクターの作製

ヒトC R T H 2（以下C R T H 2と記すこともある）のcDNAは全合成し、以降の試験に用いた。ヒトC R T H 2のcDNA配列を配列番号1に、アミノ酸配列を配列番号2に記載する。

[0273] (i) ヒトC R T H 2 遺伝子発現p K A N T E X 9 3 ベクターの構築

制限酵素E c o R I およびK p n I を用いて、ヒトC R T H 2 のc D N A をベクターp K A N T E X 9 3 (国際公開第97/10354号)と連結し、ヒトC R T H 2 遺伝子発現p K A N T E X 9 3 ベクターを構築した。

[0274] (ii) ヒトC R T H 2 遺伝子発現p A M o h ベクターの構築

p A M o h (国際公開第03/087366号)に対しても、制限酵素K p n I およびH i n d I I I を用いてヒトC R T H 2 遺伝子を組み込み、ヒトC R T H 2 遺伝子発現p A M o h ベクターを構築した。

[0275] (2) ヒトC R T H 2 発現C H O / D G 4 4 細胞の造成

D H F R 遺伝子欠損C H O (チャイニーズハムスター卵巣)細胞D G 4 4 株(C H O / D G 4 4 細胞)を、三菱化学株式会社・横浜総合研究所より入手し、ヒトC R T H 2 発現細胞造成に使用した。培養には10%透析ウシ胎児血清(d F B S) (G I B C O 社)、HT [ヒポキサンチン(H)、チミジン(T)] s u p p l e m e n t (G I B C O 社)、及び50 μ g/mL ゲンタマイシン(ナカライテスク社)を添加したI M D M (G I B C O 社)(以下、I M D M 培養培地と略記する)を用いた。

[0276] まず、(1) - (i) で作製されたヒトC R T H 2 遺伝子発現p K A N T E X 9 3 ベクターを制限酵素A a t I I 処理によって切断し、得られた直鎖状D N A を精製し、滅菌水に溶解した。このD N A をエレクトロポレーション法により、C H O / D G 4 4 細胞に導入し、HT s u p p l e m e n t を抜いたI M D M 培養培地にて3日程度培養した。

[0277] その後、10% d F B S、0.5 mg/mL G 4 1 8 (ナカライテスク社)、及び50 μ g/mL ゲンタマイシン(ナカライテスク社)を加えたI M D M (以下、I M D M 選択培地と略記する)で薬剤耐性細胞を選択した。選択した薬剤耐性細胞を、96ウェルプレートに75 c e l l s / プレートとなる様に播種し、さらに2週間ほどI M D M 選択培地にて培養した。ウェル毎に顕微鏡下で観察し、シングルクローンとなっているものを順次拡大培養した。

[0278] 得られた薬剤耐性細胞を0.02%EDTA溶液（ナカライテスク社）で剥離し、PBS（phosphate buffered saline）で洗浄した後、2%ウシ胎児血清（FBS）、0.05%NaN₃及び1mMEDTAを含むPBS（Staining Medium、以下SMと略記する）で懸濁した。次に、 $2 \times 10^5 \text{ cells/well}$ となるように96ウェルプレートに播種し、1700rpmで2分間の遠心分離を行った。

[0279] 上清を除いた後、SMで調製したPE標識抗ヒトCRTH2抗体（ベクマンコールター社）を添加し、4℃で1時間の反応を行った。細胞を洗浄した後、細胞をSMで懸濁し、蛍光強度をフローサイトメーター（BDバイオサイエンス社、FACS Cantoll）で解析した。ヒトCRTH2が高発現するクローンを選択し、この細胞をヒトCRTH2発現CHO/DG44細胞とした。

[0280] (3) FLAG融合ヒトCRTH2発現ベクターの作製

(i) FLAG融合ヒトCRTH2遺伝子発現pAMohベクターの構築

(1) - (ii) で作製されたヒトCRTH2遺伝子発現pAMohから、プライマーhumanCRTH2FLAG-A（配列番号3）及びhumanCRTH2FLAG-B（配列番号4）を用いて、C末端にFLAGタグが付加されたヒトCRTH2（配列番号5）をpolymerase chain reaction（以下、PCRと記す）で増幅し、制限酵素KpnIおよびHindIIIを用いて、ベクターpAMoh（国際公開第03/087366号）と連結し、FLAG融合ヒトCRTH2遺伝子発現pAMohベクターを構築した。

[0281] (ii) FLAG融合ヒトCRTH2遺伝子発現pKANTEX93ベクターの構築

(3) - (i) で構築されたFLAG融合ヒトCRTH2遺伝子発現pAMohベクターから、プライマーhumanCRTH2FLAG-C（配列番号6）及びhumanCRTH2FLAG-D（配列番号7）を用いて、C末端にFLAGタグが付加されたヒトCRTH2をPCRで増幅し、制限

酵素EcoRIおよびKpnIを用いて、ベクターpKANTEX93（国際公開第97／10354号）と連結し、FLAG融合ヒトCIRTH2遺伝子発現pKANTEX93ベクターを構築した。

[0282] (4) FLAG融合ヒトCIRTH2発現3Y1-B細胞の造成

ラット3Y1-B細胞を、理研バイオリソースセンターより入手し、FLAG融合ヒトCIRTH2発現細胞造成に使用した。培養には10%FBS（GIBCO社）、及び50μg/mL ゲンタマイシン（ナカライテスク社）を添加したDMEM（GIBCO社）（以下、DMEM培養培地と略記する）を用いた。

[0283] (3) - (ii) で作製されたFLAG融合ヒトCIRTH2遺伝子発現pKANTEX93ベクターを制限酵素AatII処理によって切断し、得られた直鎖状DNAを精製し、滅菌水に溶解した。このDNAを、Fugene6（プロメガ社）を用いたLipofection法により3Y1-B細胞に導入し、DMEM培養培地にて3日間程度培養した。その後、10%FBS、0.8mg/mL G418（ナカライテスク社）、及び50μg/mL ゲンタマイシン（ナカライテスク社）を添加したDMEM（以下、DMEM選択培地）で薬剤耐性細胞を選択した。

[0284] 得られた薬剤耐性細胞を0.05%トリプシン溶液（invitrogen社）で剥離し、PBSで洗浄した後、SMで懸濁した。次に、 2×10^5 cells/wellとなるように96ウェルプレートに播種し、1700rpmで2分間の遠心分離を行った。上清を除いた後、SMで調製したPE標識抗ヒトCIRTH2抗体（ベックマンコールター社）を添加し、4℃で1時間の反応を行った。細胞を洗浄した後、細胞をSMで懸濁し、蛍光強度をフローサイトメーター（BDバイオサイエンス社、FACS Aria）で解析した。FLAG融合ヒトCIRTH2を高発現する画分をシングルセルソーティングし、拡大培養を行い、FLAG融合ヒトCIRTH2発現3Y1-B細胞とした。

[0285] [実施例2]

ヒトC R T H 2に対する抗体モノクローナル抗体の作製

(1) ラットへの免疫

ヒトC R T H 2に対するモノクローナル抗体を取得するために、初回投与時の週齢が9週齢の雌性W K Y / N C r l C r l j ラット (W K Y ラット) (チャールスリバー社) に免疫した。

[0286] 初回投与は、 5×10^6 c e l l s の F L A G 融合ヒトC R T H 2 発現 3 Y 1 - B 細胞を $100 \mu\text{L}$ の生理食塩液 (大塚製薬工場社) に懸濁し、S i g m a A d j u v a n t S y s t e m (登録商標) (シグマアルドリッチ社) $100 \mu\text{L}$ と合わせて $200 \mu\text{L}$ の細胞懸濁液を調製し、1ヶ所あたり $100 \mu\text{L}$ を W K Y ラットの尾根部に左右2ヶ所筋肉内投与した。

[0287] また初回投与2週間後に、 5×10^6 c e l l s の F L A G 融合ヒトC R T H 2 発現 3 Y 1 - B 細胞を $200 \mu\text{L}$ の生理食塩液に懸濁し、同様の方法で投与を行った。

[0288] (2) ハイブリドーマの作製

(1) で2回目の免疫を行った3日後に、外科的にW K Y ラットから腸骨リンパ節を摘出し、細胞融合に供した。

[0289] まず、摘出した腸骨リンパ節をスライドガラスですりつぶし、組織をほぐした。この腸骨リンパ節組織を M i n i m u m E s s e n t i a l M e d i a (MEM) (i n v i t r o g e n 社) に懸濁し、セルストレーナーを通すことにより、余分な組織を除いた。 1200 rpm で5分間遠心分離することにより上清を除いた後、再びMEMで懸濁し、腸骨リンパ節細胞とした。

[0290] 得られた腸骨リンパ節細胞数に対し、1 / 5 細胞数のマウスミエローマ細胞株 P 3 - U 1 (A T C C) を混合した。遠心分離により上清を除いた後、 37°C の温浴の中で温めつつ、 $500 \mu\text{L}$ の P E G 溶液 [P o l y e t h y l e n e g l y c o l 1000 (純正化学社) 及びMEMをそれぞれ 1 mL ずつ混合し、DMSO (ジメチルスルホキシド) (シグマアルドリッチ社) を $350 \mu\text{L}$ 加えたもの] を穏やかに添加し、そこに1分毎にMEM

1 mLを5回加えた後さらに45 mLのMEMを添加した。

[0291] 900 rpmで5分間遠心分離することにより上清を除いた後、細胞をHAT培地[500 mLのRPMI-1640 (和光純薬社) に対し、10 mLのHAT (ヒポキサンチン (H)、アミノプテリン (A)、チミジン (T)) 溶液 (GIBCO社)、0.5 mLの55 mmol/L 2-Mercaptoethanol (invitrogen社)、50 mLのウシ胎児血清 (Moregate Biotech社)、および0.5 mLの10 mg/mL ゲンタマイシン溶液 (ナカライテスク社) を添加したもの] で懸濁し、96 ウェルプレートに播種し、培養した。

[0292] (3) ハイブリドーマスクリーニング

(2) で播種されたハイブリドーマを7日間培養した後、各ウェルの培養上清を採取し、ヒトCRTH2に対する反応性を解析した。陽性対照細胞及び陰性対照細胞は、それぞれヒトCRTH2 発現CHO/DG44細胞及びCHO/DG44細胞とした。まず、陽性対照細胞及び陰性対照細胞を0.02% EDTA溶液 (ナカライテスク社製) で剥離し、1ウェルあたりそれぞれ 1×10^5 cells/50 μ Lとなるように96ウェルプレートに播種し、50 μ Lの培養上清を添加し、4℃で30分間の反応を行った。

[0293] 細胞を洗浄した後、SMで300倍に希釈したAnti-rat IgG (Fc)-DyLight488 (abcam社) 100 μ Lで懸濁し、4℃で30分間の反応を行った。再び細胞を洗浄した後、細胞をSMで懸濁し、蛍光強度をフローサイトメトリー (BDバイオサイエンス社、FACS Cantoll) で解析した。

[0294] ヒトCRTH2 発現CHO/DG44細胞に特異的な反応が見られたウェルのハイブリドーマについて、クローニング培地[エス・クロン クローニングメデュームCM-B (エーディア社) に、0.5 mLの10 mg/mL ゲンタマイシン溶液 (ナカライテスク社)、5 mLのHT supplement (Gibco社) を添加したもの] を用いて限界希釈法によるシングルセルクローニングを2回行った。最終的に、ヒトCRTH2 発現CHO/

D G 4 4 細胞に最も強いフローサイトメトリー反応性を示すハイブリドーマ L y m 2 クローン（以下、ハイブリドーマ L y m 2 と記す）を樹立した。

[0295] （４）ハイブリドーマ L y m 2 の培養上清に含まれる抗体のサブクラスの同定

ハイブリドーマ L y m 2 を 3 日間培養した培養上清を P B S （ナカライテスク社製）で 1 0 倍に希釈し、その希釈液を 1 5 0 μ L 用いて R a t M o n o c l o n a l A n t i b o d y I s o t y p i n g T e s t K i t （A b D S e r o t e c 社）で添付の説明書に従いサブクラスの解析を行った。

[0296] その結果、ハイブリドーマ L y m 2 の培養上清中に含まれるラット抗ヒト C R T H 2 モノクローナル抗体（以下、単に L y m 2 抗体と略記する場合もある）はラット I g G 2 b 抗体であることが明らかになった。

[0297] （５）L y m 2 抗体の精製

H y b r i d o m a - S F M （i n v i t r o g e n 社）に 5 % の F e t a l B o v i n e S e r u m U l t r a L o w I g G （i n v i t r o g e n 社）を加えた培地で、ハイブリドーマ L y m 2 を 1 週間培養した。培養上清を回収し精製に供した。

[0298] 培養上清から、P r o s e p e r G （G E ヘルスケア社）を用いて、L y m 2 抗体を精製した。まず、培養上清をカラムにロードし、P B S でカラムを洗浄した後、p H 5 . 0 、 3 . 5 および 3 . 0 の溶出バッファー（0 . 1 M クエン酸一水和物 - N a O H / p H 5 . 0 、 3 . 5 および 3 . 0 ）で順に溶出した。溶出したフラクションは速やかに中和バッファー（2 M T r i s - H C l / p H 8 . 5 ）で中和した。

[0299] それぞれのフラクションの吸光度（2 8 0 n m ）を測定し、測定値の高い連続フラクションを抗体画分として回収した。P B S で透析を行い、0 . 2 2 μ m のフィルターを通したものを精製タンパク質とした。2 8 0 n m の吸光係数を 1 . 4 として、濃度を算出した。

[0300] [実施例 3]

L y m 2 抗体のフローサイトメトリーによる抗原結合性評価

F L A G 融合ヒト C R T H 2 発現 3 Y 1 - B 細胞を 0. 0 5 % トリプシン溶液 (i n v i t r o g e n 社) で剥離し、 P B S で洗浄した後、 S M で懸濁した。次に、 1 ウェルあたり 2×10^5 c e l l s となるように 9 6 ウェルプレートに播種し、 1 7 0 0 r p m で 2 分間の遠心分離を行った。上清を除いた後、 S M で $10 \mu\text{g} / \text{mL}$ になるように調製した L y m 2 抗体を $100 \mu\text{L}$ 添加し、 4°C で 1 時間の反応を行った。

[0301] 細胞を洗浄した後、 S M で 1 0 0 倍に希釈した A n t i - r a t I g G - F I T C (ベックマンコールター社) を添加し、 4°C で 1 時間の反応を行った。再び細胞を洗浄した後、細胞を S M で懸濁し、蛍光強度をフローサイトメトリー (B D バイオサイエンス社製、 F A C S C a n t o l l) で解析した。その結果、 L y m 2 抗体の F L A G 融合ヒト C R T H 2 発現 3 Y 1 - B 細胞への結合が認められた。

[0302] [実施例 4]

L y m 2 抗体重鎖及び軽鎖可変領域遺伝子のクローニング

R N A i s o p l u s (タカラバイオ社) を用いて、添付の説明書に従い、 P B S で洗浄したハイブリドーマ L y m 2 を溶解し、 t o t a l R N A を調製した。得られた t o t a l R N A は、 D E P C t r e a t e d w a t e r (i n v i t r o g e n 社) に溶解した。次に、 O l i g o t e x - d T 3 0 < S u p e r > m R N A P u r i f i c a t i o n K i t (F r o m T o t a l R N A) (タカラバイオ社) を用いて、添付の説明書に従い、得られた t o t a l R N A から m R N A を精製した。さらに、 S M A R T R A C E c D N A A m p l i f i c a t i o n K i t (C l o n t e c h 社) を用いて、添付の説明書に従い、精製した m R N A から c D N A を調製した。

[0303] 得られた c D N A を鋳型に、プライマー R a t I g G 2 b H - A (配列番号 8) 及び R a t I g G 2 b H - B (配列番号 9) を用いて、ラット I g G 2 b 重鎖遺伝子を、プライマー R a t k - A (配列番号 1 0) 及び R a t k

ーB（配列番号11）を用いて、ラット軽鎖（ κ 鎖）遺伝子を、それぞれPCRにより増幅した。増幅した遺伝子をサブクローニングし、塩基配列を解析した。

[0304] その結果、シグナル配列を含むLym2抗体のVHおよびVLの塩基配列およびアミノ酸配列を同定した。VH、VLそれぞれの塩基配列を配列番号12、14、アミノ酸配列を配列番号13、15に示した。また、Kabataら[Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services(1991)]の報告から、シグナル配列を含まないLym2抗体のVHおよびVLの配列を同定した。

[0305] シグナル配列を除いたVH、VLそれぞれの塩基配列を配列番号16、18に、アミノ酸配列を配列番号17、19に示した。また、Lym2抗体のVHのCDR1、CDR2、及びCDR3のアミノ酸配列を、それぞれ配列番号20、21、及び22に、Lym2抗体のVLのCDR1、CDR2、及びCDR3のアミノ酸配列を、それぞれ配列番号23、24、及び25に示した。

[0306] [実施例5]

ラット／ヒトキメラ型Lym2抗体の作製

(1) ラット／ヒトキメラ型Lym2抗体発現ベクターの構築

ラット／ヒトキメラ型Lym2抗体発現ベクターは、以下の方法で、Lym2抗体重鎖及び軽鎖可変領域遺伝子を、それぞれヒトIgG1重鎖及び κ 鎖定常領域遺伝子に連結することにより作製した。

[0307] まずVHとして、配列番号26の配列を、VLとして、配列番号27の配列を全合成した。合成された配列から、プライマーchLym2VH-A（配列番号28）およびプライマーchLym2VH-B（配列番号29）を用いてVHを、プライマーchLym2VL-A（配列番号30）およびプライマーchLym2VL-B（配列番号31）を用いてVLをPCRにより増幅した。

[0308] 遺伝子断片はアガロースゲル電気泳動を行い、QIAquick Gel

Extraction Kit (QIAGEN社) によって精製した。この断片と、ヒトκ鎖定常領域発現ベクター (BglII/BsiWI処理) およびヒト重鎖 (IgG1) 定常領域発現ベクター (SalI/NheI処理) を用いて、In-Fusion HD Cloning Kit (クロンテック社) によるベクターへのサブクローニングを添付の説明書に従って行った。

[0309] 大腸菌DH5αコンピテントセル (タカラバイオ社) に形質転換して、プラスミド抽出、シーケンス確認を実施することで、ラット/ヒトキメラ型Lym2抗体 (以下、chLym2と記す) 発現ベクターを作製した。

[0310] (2) chLym2一過性発現細胞株の調製

chLym2の一過性発現株を作製するため、FreeStyle (商標) MAX CHO Expression System (Life Technologies社) を、添付の説明書に従って使用し、以下の方法で、宿主細胞に (1) で作製された発現ベクターを導入した。宿主細胞には、FUT8ノックアウトCHO細胞 (国際公開第2005/035586号、国際公開第02/31140号) をFreeStyle (商標) CHO Expression Medium (Life Technologies社) に馴化した株を使用した。

[0311] (1) で作製した312.5 μgのchLym2抗体発現ベクター (軽鎖発現ベクターと重鎖発現ベクターを1:2で混合したもの) を20 mLのOpti-Pro SFM (Invitrogen社) に、また、312.5 μLのFreeStyle MAX Reagent (Invitrogen社) を20 mLのOpti-Pro SFMにそれぞれ溶解し、室温で5分間放置した。上記二液を混合し、室温で15分間放置した。該混合溶液を250 mLの宿主細胞培養液 (1×10^6 cells/mL) に全量添加しchLym2一過性発現細胞株を得た。

[0312] (3) chLym2の精製

(2) で得られたchLym2一過性発現細胞株を、8 mMのL-glucose

t a m i n e (i n v i t r o g e n 社) を添加した F r e e s t y l e
C H O e x p r e s s i o n m e d i u m (i n v i t r o g e n 社
) に懸濁し、三角フラスコで5日間培養した後、培養上清を回収した。回収
した培養上清を遠心分離し、 $0.22\mu\text{m}$ フィルターを用いてろ過すること
で c h L y m 2 を含む培養上清を調製した。

[0313] 調製した培養上清から M a b S e l e c t S u R e (G E ヘルスケア社
) を用いて、c h L y m 2 を精製した。まず、培養上清をカラムにロードし
、P B S でカラムを洗浄した後、p H 5. 0、3. 5、3. 0 の溶出バッ
ファー (0.1M クエン酸一水和物 - N a O H / p H 5. 0、3. 5、3. 0
) で順に溶出した。溶出したフラクションは速やかに中和バッファー (2M
T r i s - H C l / p H 8. 5) で中和した。

[0314] それぞれのフラクションの 280nm の吸光度 (A_{280}) を測定し、測定値
の高い連続フラクションを抗体画分として回収した。抗体画分を P B S で透
析し、 $0.22\mu\text{m}$ のフィルターを通したものを精製タンパク質とした。 280nm
の吸光係数を 1.37 として、濃度を算出した。

[0315] [実施例6]

ヒト化抗体の作製

(1) ヒト化 L y m 2 抗体重鎖及び軽鎖可変領域の設計

(i) ヒト化 L y m 2 抗体の V L および V H のアミノ酸配列の設計

ヒト化 L y m 2 抗体の V L のアミノ酸配列を以下のようにして設計した。

まず L y m 2 抗体の V L の C D R 1 ~ 3 のアミノ酸配列 (配列番号 23、
24、25) の移植に適したヒト抗体の V L の F R のアミノ酸配列を、以下
のようにして選択した。

[0316] 既知のヒト抗体重鎖可変領域配列は T h e N a t i o n a l C e n t
e r f o r B i o t e c h n o l o g y I n f o r m a t i o n が提
供する B L A S T P データベースにより、L y m 2 抗体の V L の F R 配列と
相同性の高いヒト抗体配列を検索した。その結果、G e n e B a n k I D
: A B A 7 1 3 7 4. 1 が最も相同性の高いヒト抗体配列であったため、こ

の抗体のF Rを選択した。以上のようにして決定したヒト抗体F R配列の適切な位置に配列番号23、24、25で表されるL y m 2抗体のV LのC D R 1～3のアミノ酸配列を移植することで、L V O（配列番号33）を設計した。

[0317] 次に、ヒト化L y m 2抗体のV Hのアミノ酸配列を以下のようにして設計した。L y m 2抗体のV HのC D R 1～3のアミノ酸配列（配列番号20、21、22）の移植に適したヒト抗体V HのF Rのアミノ酸配列を以下のようにして選択した。V L同様にB L A S T Pデータベースにより、L y m 2のV HのF R配列と相同性の高いヒト抗体配列を検索した。

[0318] その結果、G e n e B a n k I D : A A Y 3 3 3 3 1 . 1が最も相同性の高いヒト抗体配列であったため、この抗体のF Rを選択した。以上のようにして決定したヒト抗体F R配列の適切な位置に配列番号20、21、22で表されるL y m 2抗体のV HのC D R 1～3のアミノ酸配列を移植することで、H V O（配列番号49）を設計した。

[0319] 上記で設計したヒト化L y m 2抗体のV Lのアミノ酸配列L V O、およびV Hのアミノ酸配列H V Oは、選択したヒト抗体のF Rのアミノ酸配列に、ラットモノクローナル抗体であるL y m 2由来のC D Rのアミノ酸配列のみを移植した配列である。しかし、一般に、ヒト化抗体を作製する場合には、単にげっ歯類由来抗体のC D Rのアミノ酸配列をヒト抗体のF Rへ移植するのみでは、結合活性が低下してしまうことが多い。

[0320] このような結合活性の低下を回避するため、C D Rのアミノ酸配列の移植とともに、ヒト抗体とげっ歯類抗体で異なっているF Rのアミノ酸残基のうち、結合活性に影響を与えると考えられるアミノ酸残基を置換することが行われている。そこで、本実施例においても、結合活性に影響を与えると考えられるF Rのアミノ酸残基を以下のようにして同定し、置換した。

[0321] まず、上記で設計したヒト化L y m 2抗体のV Lのアミノ酸配列L V O、およびV Hのアミノ酸配列H V Oより構成される抗体可変領域（以下、L V O H V Oと表記する）の三次元構造をコンピューターモデリングの手法を用

いて構築した。

[0322] 三次元構造座標作製および三次元構造の表示には、Discovery Studio（アクセルリス社）を用いた。また、L y m 2 抗体の可変領域の三次元構造のコンピューターモデルも同様にして構築した。更に、L V O H V O の V L および V H の F R のアミノ酸配列の中で、L y m 2 抗体と異なっているアミノ酸残基を選択し、L y m 2 抗体のアミノ酸残基へ改変したアミノ酸配列を作製し、同様に三次元構造モデルを構築した。これら作製した L y m 2 抗体、L V O H V O および改変体の各可変領域の三次元構造を比較し、抗体の結合活性に影響を与えると予測されるアミノ酸残基を同定した。

[0323] その結果、L V O H V O の F R のアミノ酸残基の中で抗原結合部位の三次元構造を変化させ、抗体の結合活性に影響を与えると考えられるアミノ酸残基として、L V O では、配列番号 33 のアミノ酸配列の 2 番目の I l e 、4 番目の M e t 、15 番目の P r o 、および 85 番目の A l a を、H V O では、配列番号 49 のアミノ酸配列の 18 番目の L e u 、77 番目の A s n 、93 番目の V a l 、および 117 番目の T h r を、それぞれ選択した。

[0324] これらの選択したアミノ酸残基のうち、少なくとも 1 つ以上のアミノ酸配列を L y m 2 抗体の同じ部位に存在するアミノ酸残基へ置換するアミノ酸改変をし、様々な改変を有するヒト化抗体の V L および V H を設計した。具体的には、V L については、配列番号 33 のアミノ酸配列の 2 番目の I l e を V a l に、4 番目の M e t を L e u に、15 番目の P r o を L e u に、または 85 番目の A l a を P r o に置換するアミノ酸改変のうち、少なくとも 1 つの改変を導入した。V H については、配列番号 49 のアミノ酸配列の 18 番目の L e u を M e t に、77 番目の A s n を S e r に、93 番目の V a l を T h r に、または 117 番目の T h r を V a l に置換するアミノ酸改変のうち、少なくとも 1 つの改変を導入した。

[0325] L V O H V O 、または L V O H V O の F R に存在する少なくとも 1 つのアミノ酸残基を改変したヒト化 L y m 2 抗体の抗体可変領域として、L V O H V O 、L V 1 H V O 、L V 2 a H V O 、L V 2 b H V O 、L V 2 c H V O 、

L V 3 a H V 0、L V 3 b H V 0、L V 4 H V 0、L V 0 H V 4、L V 1 H V 4、L V 2 a H V 4、L V 2 b H V 4、L V 2 c H V 4、L V 3 a H V 4、L V 3 b H V 4、L V 4 H V 4、L V 0 H V 1、L V 0 H V 2 a、L V 0 H V 2 b、および、L V 0 H V 3をそれぞれ設計した。

[0326] 以降の記述においては、上述の可変領域を含むヒト化L y m 2抗体をそれぞれL V 0 H V 0、L V 1 H V 0、L V 2 a H V 0、L V 2 b H V 0、L V 2 c H V 0、L V 3 a H V 0、L V 3 b H V 0、L V 4 H V 0、L V 0 H V 4、L V 1 H V 4、L V 2 a H V 4、L V 2 b H V 4、L V 2 c H V 4、L V 3 a H V 4、L V 3 b H V 4、L V 4 H V 4、L V 0 H V 1、L V 0 H V 2 a、L V 0 H V 2 b、および、L V 0 H V 3と略記する。

[0327] 軽鎖可変領域L V 0（配列番号33）、L V 1（配列番号35）、L V 2 a（配列番号37）、L V 2 b（配列番号39）、L V 2 c（配列番号41）、L V 3 a（配列番号43）、L V 3 b（配列番号45）、L V 4（配列番号47）、および重鎖可変領域H V 0（配列番号49）、H V 1（配列番号51）、H V 2 a（配列番号53）、H V 2 b（配列番号55）、H V 3（配列番号57）、H V 4（配列番号59）のアミノ酸配列をそれぞれ図1および図2に示す。

[0328] (i i) ヒト化L y m 2抗体の可変領域遺伝子の設計

ヒト化抗体の可変領域のアミノ酸配列をコードする塩基配列は、動物細胞で高頻度で使用するコドンを用いて設計した。これら塩基配列を用いて、以下に示すヒト化L y m 2抗体発現ベクターの構築および対応する抗体の発現を行った。

[0329] (2) ヒト化L y m 2抗体発現ベクターの構築

実施例5-(1)に記載の方法に準じて、ヒト化L y m 2抗体発現ベクターを構築した。すなわち、配列番号33、35、37、39、41、43、45、47、49、51、53、55、57および59で示されるアミノ酸配列をそれぞれコードする、配列番号32、34、36、38、40、42、44、46、48、50、52、54、56および58で示される塩基配

列のDNAを全合成し、配列番号28-31で示すプライマーにより、対応するVL、VH遺伝子断片をPCRによって増幅した。遺伝子断片はアガロースゲル電気泳動を行い、QIAquick Gel Extraction Kit (QIAGEN社)によって精製した。

[0330] このVLまたはVH断片を、In-Fusion HD Cloning Kit (クロンテック社)を用いて添付の説明書に従い、それぞれヒトκ鎖定常領域発現ベクター (BglII/BsiWI処理) およびヒト重鎖 (IgG1) 定常領域発現ベクター (SalI/NheI処理) ヘサブクロニングした。作製したベクターを用いて大腸菌 DH5αコンピテントセル (タカラバイオ社) を形質転換して、プラスミド抽出、シーケンス確認を実施し、正しい配列が挿入されたコロニーを選抜し、一過性発現のためにプラスミド大量調製を行った。

[0331] (3) ヒト化Lym2抗体の一過性発現

作製したヒト化Lym2抗体の一過性発現は、実施例5-(2)に記載のFreeStyle (商標) CHO Expression Medium (Life technologies社) に馴化したCHO細胞を宿主とし、FreeStyle (商標) MAX CHO Expression System (Life technologies社) を使用して行った。プラスミド導入の方法は添付の説明書に従った。軽鎖発現ベクターと重鎖発現ベクターは、1:2の比率で混合して使用した。

[0332] 培養液量は200mLで行い、37℃、8%CO₂、125rpmの設定条件下で、5日間培養した。培養後、細胞懸濁液の遠心分離を行い、0.2μmフィルター (ThermoScientific社) を通してヒト化Lym2抗体を含む培養上清を回収した。

[0333] (4) ヒト化Lym2抗体の精製

以下に示す、MabSelect SuRe (GE Healthcare社) を用いたアフィニティー精製により、ヒト化Lym2抗体を精製した。レジンをPBSで平衡化した後、(3) で取得された培養上清をロードし

、PBSで2回洗浄した。

[0334] 洗浄後、溶出バッファー（20mM クエン酸、50mM NaCl、pH 3.4）を用いて抗体を溶出し、中和バッファー（1M リン酸-NaOH、pH 7.0）を1/10量加えて中和した。続いてNAP25（GE Healthcare社）を用いてPBSにバッファー置換を行った。Amicon Ultra-4 Centrifugal Filter Units（ミリポア社）を用いて限外濾過による濃縮を行い、Nanodrop 8000（Thermo Scientific社）を使用して280nmにおける吸光度（ A_{280} ）を測定し、抗体溶液の濃度測定および調製を行った。

[0335] [実施例7]

chLym2およびヒト化Lym2抗体の抗原結合活性

FLAG融合ヒトCRTM2発現3Y1-B細胞を0.25%Trypsin-EDTA（ナカライテスク社）で剥離し、PBSで洗浄した後、SMで懸濁した。次に、細胞数を1ウェルあたり 1×10^5 個となるように96ウェルプレートに播種し、50000、12500、3125、781、195、49、12および3ng/mL各最終濃度のchLym2またはヒト化Lym2抗体を添加し、4℃で60分間の反応を行った。

[0336] 細胞をSMで洗浄した後、SMで500倍に希釈したGoat F(ab')₂ Anti-Human IgG PE (γ chain specific)（Southern Biotech社）を添加し、4℃で60分間の反応を行った。SMで細胞を洗浄した後、細胞を50μLのSMで再懸濁し、蛍光強度をフローサイトメトリー（BDバイオサイエンス社、FACS Cantoll）で測定した。

[0337] データはFlowJo 7.65（トミーデジタルバイオロジー社）によって解析し、各濃度におけるGeomean値からLogistic曲線によるカーブフィッティングを行い、統計解析言語R（Ver. 3.02）を用いてchLym2および各ヒト化Lym2抗体の結合の50%effec

tive concentration (EC_{50}) 値およびその standard error (SE) 値を算出した。結果を表 1 に示す。

[0338] [表1]

No.	mAb	EC_{50} value ($\mu\text{g/mL}$)	No.	mAb	EC_{50} value ($\mu\text{g/mL}$)
1	chLym2	0.54 ± 0.072	13	chLym2	0.50 ± 0.077
2	LV0HV0	0.87 ± 0.052	14	LV2bHV4	0.59 ± 0.038
3	LV1HV0	0.69 ± 0.057	15	LV2cHV4	0.54 ± 0.070
4	LV2aHV0	0.66 ± 0.053	16	LV3aHV4	0.76 ± 0.135
5	LV2bHV0	0.67 ± 0.061	17	LV3bHV4	0.53 ± 0.095
6	LV2cHV0	0.68 ± 0.071	18	LV4HV4	0.68 ± 0.116
7	LV3aHV0	0.70 ± 0.078	19	LV0HV0	0.55 ± 0.052
8	LV3bHV0	0.81 ± 0.050	20	LV0HV1	0.78 ± 0.088
9	LV4HV0	0.74 ± 0.076	21	LV0HV2a	0.65 ± 0.077
10	LV0HV4	0.58 ± 0.052	22	LV0HV2b	0.72 ± 0.064
11	LV1HV4	0.58 ± 0.045	23	LV0HV3	0.69 ± 0.032
12	LV2aHV4	0.51 ± 0.068	24	LV0HV4	0.67 ± 0.023

[0339] その結果、表 1 に示すように、各種ヒト化抗体がキメラ抗体と同等のヒト C R T H 2 反応性を有することが示唆された。

[0340] [実施例 8]

chLym2 およびヒト化 L y m 2 抗体の好酸球、好塩基球に対する除去 (depletion) 活性

ヘパリンナトリウム注射液を加えて採取したヒト末梢血を 4°C 、 1500rpm 、30 分間遠心分離し、血漿を回収した。

[0341] 血漿を回収した後の赤血球を含むペレットに、PBS (ナカライテスク社) を加えて元の血液容量に戻し、懸濁したもの 1mL に対して、溶血用溶液 [$10\times\text{RBC Lysis buffer}$ (e-bioscience 社) を滅菌水で 10 倍に希釈したもの] を 10mL 添加して転倒混和した。室温で 10 分間放置した後、常温、 1500rpm 、5 分間の遠心分離を行い、上清を除き、PBS で 2 回洗浄した。

[0342] その後、細胞ペレットを、回収していた血漿で懸濁し、 $300\mu\text{L}/\text{well}$ で 48well プレートに播種し、 1000 、 100 、 33 、 11 、 3 、 7 、 1 、 2 、 0.4 および $0.01\text{ng}/\text{mL}$ 各最終濃度の chLym2

、各ヒト化L y m 2抗体L V O H V 0、L V O H V 1、L V O H V 2 a、またはアイソタイプコントロール抗体 [Clin Cancer Res 2005, 11(8), 3126-3135記載の抗2, 4-d i n i t r o p h e n o l (DNP) I g G 1抗体をコードするベクターを用い、実施例5に記載の方法に準じて作製したI g G 1抗体（以下、抗DNP I g G 1抗体と記す）] を添加し、37℃、5% CO₂インキュベーター内で、20時間反応させた。

[0343] 反応後、各w e l lの細胞液を回収し、SMを10mL添加し、コントロールビーズとしてCountBright Absolute Counting Beads、for flow cytometry (Molecular Probes社)を200μL／サンプルで添加した後、4℃、2000rpm、10分間の遠心分離を行い、上清を除いた。SMで2回洗浄し、SMで希釈した10000μg/mL I g G from human serum (シグマアルドリッチ社)を300μL／サンプルで添加して、懸濁した後、4℃、30分間反応させた。

[0344] その後、40μL／w e l lで96ウェルプレートに播種し、好酸球の検出として、PE anti-human Siglec-8 Antibody (BioLegend社)、好塩基球の検出としてPE-Cy7 Mouse Anti-Human CD123 (BDバイオサイエンス社)およびAnti-Human Fc epsilon Receptor 1 alpha (FcεR1) APC (eBioscience社)を、それぞれ5μL／w e l l添加し、4℃、40分間反応させた。

[0345] SMで2回洗浄後、7-AAD Staining Solution (BDバイオサイエンス社)を1%含有したSMで細胞を懸濁し、4℃、10分間放置した後、蛍光強度をフローサイトメトリー (BDバイオサイエンス社製、FACS Cantoll)を用いて解析した。

[0346] ヒト好酸球は、FSC-SSC展開の顆粒球画分における7-AAD陰性 siglec8-PE陽性画分として検出した。ヒト好塩基球は、FSC-SSC展開のリンパ球画分における7-AAD陰性、CD123-PC-C

y 7 陽性、F c ε R 1 - A P C 陽性画分として検出した。細胞除去活性は、一定数のC o u n t B r i g h t のc o u n t 数当たりのそれぞれの細胞のc o u n t 数を解析することで評価した。

[0347] その結果、図3 (A) ~ 図3 (C) に示すように、評価したヒト化L y m 2 抗体L V O H V 0、L V O H V 1 およびL V O H V 2 a はいずれも、キメラL y m 2 抗体c h L y m 2 と同等の好酸球および好塩基球に対する細胞傷害活性を示すことが明らかとなった。

[0348] [実施例9]

比較対照用抗ヒトC R T H 2 抗体の作製

(1) 比較対照用抗ヒトC R T H 2 抗体発現ベクターの作製

国際公開第2014/144865号記載の抗ヒトC R T H 2 モノクローナル抗体h u 1 9 A 2 v 5 2、h u 8 B 1 v 1、m u 8 B 1、m u 3 C 1 2 およびm u 3 1 A 5 のV H およびV L のアミノ酸配列（それぞれ、国際公開第2014/144865号中ではS E Q I D N O s : 5 7 および4 0、6 4 および5 2、6 2 および5 0、6 3 および5 1、並びに6 5 および5 3 で表される）をコードする塩基配列を、全合成した。

[0349] 実施例5 - (1) に記載の方法に準じて、それぞれの抗体のV H およびV L の組み合わせとなるよう、上記塩基配列を抗体発現ベクターに組み込み、5 種の比較対照用ヒト化またはキメラ抗ヒトC R T H 2 抗体（それぞれh u 1 9 A 2 v 5 2、h u 8 B 1 v 1、c h 8 B 1、c h 3 C 1 2 およびc h 3 1 A 5）発現ベクターをそれぞれ作製した。

[0350] (2) 比較対照用抗ヒトC R T H 2 抗体の一過性発現細胞の作製および抗体の精製

実施例5 - (2) および(3) に記載の方法に準じて、h u 1 9 A 2 v 5 2、h u 8 B 1 v 1、c h 8 B 1、c h 3 C 1 2 またはc h 3 1 A 5 の抗体発現ベクターを宿主細胞に一過性発現させ、培養上清からそれぞれの抗体の精製を行った。

[0351] [実施例10]

ヒトC R T H 2 アミノ酸置換体発現細胞を用いた、抗ヒトC R T H 2 モノクローナル抗体のエピトープ解析

(1) ヒトC R T H 2 アミノ酸置換体発現ベクターの作製

ヒトC R T H 2 のアミノ酸配列のうち、細胞外領域のアミノ酸残基を部分的に別のアミノ酸残基に置換したアミノ酸置換体の発現ベクターを作製した。具体的には、配列番号2で表されるアミノ酸配列にS 2 A ; N 4 A ; T 6 A およびL 7 A ; K 8 A、P 9 A およびL 1 0 A ; P 1 2 A、L 1 4 A およびE 1 5 A ; Q 1 6 E、R 1 9 H およびQ 2 1 R ; H 2 3 A、S 2 4 A およびN 2 5 A ; T 2 6 A、S 2 7 A およびI 2 8 A ; D 1 7 1 A、T 1 7 2 A およびI 1 7 3 A ; S 1 7 4 A、R 1 7 5 A およびL 1 7 6 A ; D 1 7 7 A、G 1 7 8 A およびR 1 7 9 A ; I 1 8 0 A およびM 1 8 1 A ; Y 1 8 3 A、Y 1 8 4 A およびN 1 8 5 A ; L 1 8 7 A、L 1 8 8 A およびL 1 8 9 A ; N 1 9 0 A ; P 1 9 1 A ; G 1 9 2 A ; P 1 9 3 A ; D 1 9 4 A ; R 1 9 5 A ; D 1 9 6 A およびT 1 9 8 A ; N 2 7 5 A、G 2 7 7 A およびL 2 7 8 A ; P 2 7 6 A ; P 2 7 9 A およびL 2 8 1 A ; P 2 8 0 A ; またはV 2 8 2 A、R 2 8 3 A およびR 2 8 4 A の置換をしたアミノ酸置換体を発現するベクターをそれぞれ作製した。なお、前記アミノ酸置換の記号は「置換前のアミノ酸残基の1文字表記」「N末端から数えた置換の位置」「置換後のアミノ酸残基の1文字表記」を表す。

[0352] 発現ベクターとしては、p h m A G 1 - M N L i n k e r (M B L 社) を用い、B a m H I およびH i n d I I I の制限酵素サイトに終始コドンを含失させた各種アミノ酸置換体をコードする遺伝子を挿入することで、細胞内C末端領域にアザミグリーンのタグが付加されるようにした。

[0353] 上記のアミノ酸置換体を発現するベクターは、1) アミノ酸置換体をコードする遺伝子配列を全合成し、ベクターに挿入する方法または、2) 配列番号1で示される野生型ヒトC R T H 2 アミノ酸配列をコードするDNAが挿入されたベクターから、部位特異的変異導入を行うことにより作製した。

[0354] (2) ヒトC R T H 2 アミノ酸置換体一過性発現細胞の造成

ヒトC R T H 2 アミノ酸置換体の発現細胞株の調製には、C H O - S 細胞 (L i f e T e c h n o l o g i e s 社) を用いた。細胞の継代には 8 m M L - G l u t a m i n e (i n v i t r o g e n 社) を含む F r e e s t y l e C H O e x p r e s s i o n m e d i u m (i n v i t r o g e n 社) を使用し、37℃、5%CO₂条件下で振とう培養した。

[0355] (1) で作製された 25 μg のヒトC R T H 2 アミノ酸置換体発現ベクターを 400 μL の O p t i - P r o S F M (i n v i t r o g e n 社) に、また、25 μL の F r e e s t y l e M A X R e a g e n t (i n v i t r o g e n 社) を 400 μL の O p t i - P r o S F M にそれぞれ溶解し、室温で 5 分間放置した。上記二液を混合し、室温で 15 分間放置した。該混合溶液を上記C H O - S 培養液に添加し、24 時間培養することでヒトC R T H 2 アミノ酸置換体発現細胞株を得た。

[0356] (3) ヒトC R T H 2 アミノ酸置換体発現細胞を用いた、取得抗体の反応性解析

(2) で樹立されたヒトC R T H 2 アミノ酸置換体発現C H O - S 細胞を、S M で洗浄した後、1 ウェルあたり 2×10^5 c e l l s となるように 96 ウェルプレートに播種し、1700 r p m で 2 分間の遠心分離を行った。上清を除いた後、S M で 10 μg / m L になるように調製した c h L y m 2、L V O H V 1、実施例 9 で作製された抗ヒトC R T H 2 抗体 h u 19 A 2 v 5 2、h u 8 B 1 v 1、c h 3 C 1 2 もしくは c h 3 1 A 5、または市販ラット抗ヒトC R T H 2 抗体 B M 1 6 (s a n t a c r u z 社) を添加し、4℃で 1 時間の反応を行った。

[0357] 細胞を洗浄した後、c h L y m 2、L V O H V 1、h u 19 A 2 v 5 2、h u 8 B 1 v 1、c h 3 C 1 2 または c h 3 1 A 5 が添加されたウェルには S M で希釈した g o a t a n t i - H u m a n I g G a l e x a 6 4 7 (M o l e c u l a r P r o b e s 社) を、B M 1 6 が添加されたウェルには S M で希釈した g o a t a n t i - R a t I g G a l e x a 6 4 7 (M o l e c u l a r P r o b e s 社) をそれぞれ最終濃度 10 μ

g/mLで添加し、4℃で1時間の反応を行った。

[0358] 反応後、細胞を洗浄し、細胞をSMで懸濁し、蛍光強度をフローサイトメトリー（BDバイオサイエンス社、FACS Canto II）で解析した。解析に関しては、全細胞からアザミグリーンの陽性細胞をgatingし、その集団における各抗体の反応性を解析した。

[0359] また、各変異体間での発現レベルを補正するため、各抗体の結合によるAlexa 647の蛍光強度を、C末端領域に付加したアザミグリーンの蛍光強度で除して相対的蛍光強度を算出し、各抗体の反応性とした。そして、野生型ヒトCRTH2発現細胞に対する各抗体の蛍光強度を100%とした際の、ヒトCRTH2アミノ酸置換体発現細胞への相対的蛍光強度を、各抗体に関して解析した。

[0360] その結果、図4～図7に示すように、ヒト化Lym2抗体LV0HV1は、P12A、L14AおよびE15A；D177A、G178AおよびR179A；I180AおよびM181A；Y183A、Y184AおよびN185A；L187A、L188AおよびL189A；G192A；D194A；R195A；またはD196AおよびT198AのヒトCRTH2アミノ酸置換体発現細胞への反応性が消失することが明らかになった。

[0361] また、キメラLym2抗体chLym2は、加えて、D171A、T172AおよびI173AのヒトCRTH2アミノ酸置換体発現細胞への反応性が消失することが明らかになった。ヒト化Lym2抗体LV0HV1のD171A、T172AおよびI173Aを含むヒトCRTH2アミノ酸置換体発現細胞への反応性は、大幅に低下していることから、LV1HV0およびchLym2はいずれも同じエピトープに結合していることが示唆された。

[0362] 一方、今回比較した他の抗ヒトCRTH2抗体は、本発明の抗ヒトCRTH2抗体とは異なる部位のヒトCRTH2アミノ酸置換体発現細胞への反応性が顕著に低下していたことから、既存の他の抗ヒトCRTH2抗体は、本願発明の抗体とは異なるエピトープを認識していることが明らかになった。

[0363] 更に、各アミノ酸置換体発現細胞への反応性を詳細に確認した結果、既存

の抗ヒトC R T H 2抗体はいずれもG 1 9 2 AまたはD 1 9 4 Aを含むアミノ酸置換体発現細胞への反応性は低下せず、本願発明の抗体のみが、該細胞への反応性が低下したことから、本願発明の抗ヒトC R T H 2抗体は、ヒトC R T H 2のG l y 1 9 2およびA s p 1 9 4の少なくとも1つアミノ酸残基を含むエピトープに結合することが明らかになった。

[0364] [実施例11]

ヒト好酸球に対する反応性評価

ヘパリンナトリウム注射液を加えて採取したヒト末梢血に対し、等量の注射用生理食塩水を添加し、混和した。50mL容量の遠沈管にF i c o l l - P a q u e P R E M I U M 1. 0 8 4 (GEヘルスケア社)を15mL添加し、生理食塩水で希釈した上記ヒト末梢血を30mL重層し、室温、1500rpmで30分間遠心分離を行った。

[0365] 遠心分離後、血漿層、単核球層およびF i c o l l 層の半量をアスピレーターで除去し、並びに遠心間の壁面に付着している血小板を滅菌した綿棒を用いて除いた。その後、滅菌した氷冷水27mLを各チューブに分注し30秒間溶血させた。

[0366] その後、氷冷した10×P I P E S緩衝液[500mLの蒸留水に、32.15gの塩化ナトリウム(和光純薬社)、1.85gの塩化カリウム(ナカライテスク社)、38gのP I P E S (p i p e r a z i n e - 1, 4 - b i s (2 - e t h a n e s u l f o n i c a c i d ナカライテスク社)、8.4gの水酸化ナトリウム(ナカライテスク社)を溶解させたもの]を3mL添加して等張に戻し、4℃、1200rpm、5分間遠心分離した。遠心分離終了後、上清を吸引し、2mLの1×P I P E S緩衝液[蒸留水を用いて10×P I P E S緩衝液を10倍に希釈した溶液]を加えて細胞ペレットをほぐした。

[0367] 再度上記溶血操作を繰り返し、得られた細胞ペレットを、a u t o M A C S R i n s i n g S o l u t i o n (ミルテニーバイオテク社)にM A C S B S A S t o c k S o l u t i o n (ミルテニーバイオテク社)

を添加したもの（以下、MACSバッファと略記する）を用いて2回洗浄した。

[0368] 細胞数をカウントした後、CD16 MicroBeads、human（ミルテニーバイオテク社）を用いて、添付の説明書に従い好酸球をネガティブセレクション法により単離した。

[0369] 単離された細胞をSMで洗浄した後、1ウェルあたり 1×10^5 cellsとなるように96ウェルプレートに播種し、2000 rpmで2分間の遠心分離を行った。上清を除いた後、SMで希釈した、10、3.3、1.1、0.37、および0.12 $\mu\text{g}/\text{mL}$ 各最終濃度のLym2抗体、市販CRTH2抗体のBM16（santa cruz社）または301108（R&D社）を添加し、4℃で1時間の反応を行った。

[0370] 細胞を洗浄した後、Lym2抗体またはBM16が添加されたウェルには、SMで希釈したCELL LAB Mouse Anti-Rat Kappa（kappa light chain specific）FITC（ベクマンコールター社）を、301108が添加されたウェルには、SMで希釈したCELL LAB Goat Anti-Mouse IgG（ γ chain specific）Fluorescein（FITC）Conjugate（ベクマンコールター社）をそれぞれ最終濃度10 $\mu\text{g}/\text{mL}$ で添加し、4℃で1時間の反応を行った。再び細胞を洗浄した後、細胞をSMで懸濁し、蛍光強度をフローサイトメトリー（BDバイオサイエンス社製、FACS Cantoll）で解析した。

[0371] その結果、ラット抗ヒトCRTH2抗体Lym2はヒト好酸球に対して、市販のラット抗ヒトCRTH2抗体BM16およびマウス抗ヒトCRTH2抗体301108よりも強い反応性を示すことが明らかとなった（図8）。

[0372] [実施例12]

ヒト好塩基球に対するchLym2の反応性

ヘパリンナトリウム注射液を加えて採取したヒト末梢血に対し、等量の注射用生理食塩水を添加し、混和した。50 mL容量の遠沈管にFicoll

ーPaque PREMIUM 1.084 (GEヘルスケア社)を15 mL添加し、生理食塩水で希釈したヒト末梢血を30 mL重層し、室温、1500 rpmで30分間遠心分離を行った。

[0373] 遠心分離後、単核球層をピペットで回収し、MACSバッファーを用いて2回洗浄した。細胞数をカウントした後、Basophil Isolation Kit II, human (ミルテニーバイオテク社製)を用いて、添付の説明書に従い好塩基球をネガティブセレクション法により単離した。

[0374] 単離された細胞をSMで洗浄した後、1ウェルあたり 5×10^4 cellsとなるように96ウェルプレートに播種し、2000 rpmで2分間の遠心分離を行った。上清を除いた後、100 μ LのSMで細胞を懸濁し、Zenon Alexa Fluor 647 Human IgG Labeling Kit (Molecular Probes社製)を用いて添付の説明書に従い標識を行った、chLym2または実施例8記載のアイソタイプコントロール抗体を、最終濃度10 μ g/mLになるように添加し、4°Cで1時間の反応を行った。細胞を洗浄した後、細胞をSMで懸濁し、蛍光強度をフローサイトメトリー (BDバイオサイエンス社、FACS Canto II) で解析した。

[0375] その結果、ヒト好塩基球に対して、chLym2の反応性が確認された (図9)。従って、本願発明のキメラ抗ヒトCRT2抗体cLym2は、好塩基球に結合することが明らかになった。

[0376] [実施例13]

ヒトCD4陽性T細胞に対するヒト化Lym2抗体の反応性

実施例12に準じ、Ficoll-Paque PREMIUM 1.084の代わりにFicoll-Paque PREMIUM (GEヘルスケア社)を用いて調製した単核球層の細胞懸濁液を100 μ L/wellで96ウェルプレートに播種し、EZ-Link Sulfo-NHS-LC-Biotin, No-Weigh Format (PIERCE社)を用い

て添付の説明書に従いビオチン標識したヒト化L y m 2 抗体L V O H V 1 を最終濃度 $10 \mu\text{g}/\text{mL}$ になるように添加し、 4°C で1時間の反応を行った。

[0377] 細胞を洗浄した後、SMで希釈した最終濃度 $10 \mu\text{g}/\text{mL}$ streptavidin、および添付の説明書に記載された量のAlexa Fluor 647 conjugate (Molecular Probes社)、FITC anti-human CD3 Antibody (バイオレジェンド社) およびCD4 Antibody、Clone SK3、PE-Conjugated (stem cell technology社) を添加し、 4°C で1時間の反応を行った。

[0378] 再び細胞を洗浄した後、細胞をSMで懸濁し、蛍光強度をフローサイトメトリー (BDバイオサイエンス社、FACS Cantor II) で解析した。解析は、FSC-SSC展開によりリンパ球を分画し、さらにCD3陽性かつCD4陽性細胞で分画した細胞群に対して、ヒト化L y m 2 抗体L V O H V 1 による蛍光染色の蛍光強度を縦軸に、CD4抗体による蛍光染色の蛍光強度を横軸に示すことで、CD3陽性CD4陽性細胞におけるヒト化L y m 2 抗体L V O H V 1 の反応性画分の割合を算出した。その結果、CD3陽性CD4陽性のT細胞画分の約2~3%にヒト化L y m 2 抗体L V O H V 1 が反応した (図10)。

[0379] [実施例14]

好酸球、好塩基球に対する細胞傷害活性

実施例8と同様にして調製した細胞懸濁液を、 $95 \mu\text{L}/\text{well}$ で96 wellプレートに播種し、1000、10、3、1、0.3、0.1 ng/mL各最終濃度の被験抗体を添加し、 37°C 、5% CO_2 インキュベーター内で20時間反応させた。被験抗体としては、ヒト化L y m 2 抗体L V O H V 1、実施例9で作製された抗ヒトC R T H 2 抗体hu19A2 v52、hu8B1 v1、ch3C12およびch31A5を用いた。アイソタイプコントロール抗体としては抗DNP IgG1抗体を用いた。

[0380] 反応後の各wellにSMを100 μ L/wellで添加し、コントロールビーズとしてCountBright Absolute Counting Beads, for flow cytometry (Molecular Probes社)を20 μ L/wellで添加し、4℃、2000 rpm、2分間の遠心分離を行い、上清を除いた。SMで2回洗浄し、SMで希釈した10000 μ g/mL IgG from human serum (シグマアルドリッチ社)を100 μ L/wellで添加して、4℃、30分間反応させた。以降は、実施例8と同様の方法で行った。

[0381] その結果、ヒト化抗ヒトCRTH2抗体LV0HV1は、抗体濃度依存的に好酸球および好塩基球のいずれの細胞も除去した。既存の抗ヒトCRTH2抗体ch3C12は、ヒト化抗ヒトCRTH2抗体LV0HV1と比べて細胞を除去する活性が弱かった[図11(A)および図11(B)]。従って、本願発明の抗ヒトCRTH2抗体は、CRTH2が発現する好酸球または好塩基球を標的とした治療効果を発揮し得ることが示唆される。

[0382] [実施例15]

Th2細胞に対する細胞傷害活性

実施例13と同様にして調製した単核球層を、1/10 volume FBS (Gibco社)、200mM-L-Glutamine Stock Solution (ナカライテスク社)、10mM MEM Non-Essential Amino Acids Solution (Gibco社)、100mM Sodium Pyruvate (Gibco社)、1M HEPES Buffer Solution (Gibco社) および1/100 volume Penicillin-Streptomycin, Liquid (Gibco社)を添加したRPMI1640 (和光純薬社) [以下、PBMC (peripheral blood mononuclear cell) 培養培地と記す] で2回洗浄し、 1×10^7 cells/mL細胞懸濁液を調製した。

[0383] 調製した細胞懸濁液を100 μ L/wellで96ウェルプレートに播種

し、c h L y m 2 抗体、ヒト化抗ヒトC R T H 2 抗体L V O H V 1 または実施例8記載のアイソタイプコントロール抗体を最終濃度10 μ g/mL添加し、37℃、5%CO₂インキュベーター内で20時間反応させた。

[0384] 反応後の細胞をP B M C 培養培地で7回洗浄し、被験抗体を十分に除去した後、100 μ LのP B M C 培養培地に懸濁し、抗C D 3 抗体O K T 3 (A b c a m 社)を1 μ g/mLの濃度で固相化したプレートに播種した。その後、2 μ g/mLに調製した抗C D 2 8 抗体(B D バイオサイエンス社)を100 μ L/w e l l で添加し、37℃、5%CO₂インキュベーター内で3日間反応させた。

[0385] 反応後の上清を回収し、H u m a n I F N - γ F l e x S e t (B D バイオサイエンス社)、H u m a n I L - 5 F l e x S e t (B D バイオサイエンス社)、H u m a n I L - 1 3 F l e x S e t (B D バイオサイエンス社)を用いて、添付の説明書に従い上清中サイトカインの定量を行った。

[0386] その結果、キメラ抗ヒトC R T H 2 抗体c h L y m 2 およびヒト化抗ヒトC R T H 2 抗体L V O H V 1 は、T h 2 サイトカインであるI L - 5 およびI L - 1 3 の産生を減少させたが、その一方で、T h 1 サイトカインであるI F N - γ の産生は変化させなかった。即ち、本発明の抗体はT h 2 細胞を選択的に除去していることが示唆された[図12(A)および図12(B)]。従って、本発明の抗ヒトC R T H 2 抗体は、T h 2 細胞を標的とした治療効果を発揮できることが示唆された。

[0387] [実施例16]

リガンド存在下における反応性評価(293EBNA)

実施例1-(1)-(i i)で作製したヒトC R T H 2 遺伝子発現p A M o h ベクターを、F u g e n e 6 (プロメガ社)を用いて293EBNA細胞(i n v i t r o g e n 社)に導入し、10%F B S、0.25mg/mL G 4 1 8 (ナカライテスク社)、100 μ g/mL ペニシリン、100 U/mL ストレプトマイシン(ナカライテスク社)および300 μ g/

mL ハイグロマイシンB（和光純薬社）DMEMからなる培地で薬剤耐性細胞を選択し、ヒトC R T H 2 発現293EBNA細胞を樹立した。

[0388] ヒトC R T H 2 発現293EBNA細胞を0.02%EDTA溶液（ナカライテスク社）で剥離し、PBSで洗浄した後、上記培地で懸濁した。次に、 $1 \times 10^5 \text{ cells} / 90 \mu\text{L} / \text{well}$ となるように96ウェルプレートに播種し、DKPGD2（Cayman chemical社）を最終濃度 $10 \mu\text{M}$ になるように添加した。

[0389] 37°C 、5%CO₂インキュベーター内で15分間放置した後、0.3、1および $3 \mu\text{g} / \text{mL}$ 各最終濃度のヒト化L y m 2 抗体LV0HV1、公知の抗ヒトC R T H 2 抗体hu19A2 v52、hu8B1 v1、ch3C12、ch31A5、BM16（santa cruz社）または301108（R&D社）を添加し、常温で30分間の反応を行った。

[0390] 細胞をSMで5回洗浄した後、LV0HV1、hu19A2 v52、hu8B1 v1、ch3C12またはch31A5が添加されたウェルにはSMで希釈した $10 \mu\text{g} / \text{mL}$ goat anti-Human IgG Alexa647（Molecular Probes社）を、BM16が添加されたウェルにはSMで希釈した $10 \mu\text{g} / \text{mL}$ goat anti-Rat IgG Alexa647（Molecular Probes社）を、301108が添加されたウェルには、SMで希釈した $10 \mu\text{g} / \text{mL}$ goat anti-Mouse IgG Alexa647（Molecular Probes社）をそれぞれ最終濃度 $10 \mu\text{g} / \text{mL}$ で添加し、 4°C 、40分間の反応を行った。再び細胞を洗浄した後、細胞をSMで懸濁し、蛍光強度をフローサイトメトリー（BDバイオサイエンス社、FACS Cantoll）で解析した。

[0391] その結果、図13（A）～（C）に示すように、既存の抗ヒトC R T H 2 抗体は、いずれもヒトC R T H 2 リガンドであるDKPGD2存在下で、ヒトC R T H 2 発現細胞への結合が低下したのに対して、ヒト化L y m 2 抗体LV0HV1は、検討したいずれの抗体濃度においても、ヒトC R T H 2 発

現細胞への結合は殆ど低下しなかった。

[0392] 即ち、本発明の抗体は、高濃度のリガンド存在下でも高い反応性を有することが明らかになった。従って、本発明の抗ヒトC R T H 2抗体は、リガンド存在下でもヒトC R T H 2発現細胞に対して、リガンド非存在下での反応性と同等に結合することができ、該ヒトC R T H 2発現細胞に作用することができる有用な抗体であることが示唆された。

[0393] [実施例17]

分化誘導ヒトマスト細胞に対する反応性解析

Nature Protocols 2006, 1(4), 2178-2183記載の方法に準じてマスト細胞を調製した。分化誘導開始から11週時点のマスト細胞をSMで洗浄し、1ウェルあたり 5×10^4 cellsとなるように96ウェルプレートに播種し、PE anti-human CD203c (E-NPP3) Antibody (BioLegend社製)、Brilliant Violet 421 (商標) anti-human CD117 (c-kit) Antibody (BioLegend社製)、Anti-Human Fc epsilon Receptor 1 alpha (FcεR1) PE (eBioscience社製)を添加し、4℃で1時間の反応を行った。細胞を洗浄した後、SMで懸濁し、蛍光強度をフローサイトメトリー (BDバイオサイエンス社製、FACS Cantor II) で解析した。

[0394] その結果、分化誘導を行ったマスト細胞は、マスト細胞表面マーカであるCD203c、CD117、FcεR1をいずれも発現していることが確認できた。

[0395] 分化誘導開始から15週間時点のマスト細胞培養液に、抗DNP IgG1抗体の定常領域をIgE型に組み換えたヒトIgEを最終濃度 $10 \mu\text{g}/\text{mL}$ で添加し、37℃、5%CO₂インキュベーター内で3日間反応させた。

[0396] 反応後のマスト細胞を最終濃度 $100 \mu\text{g}/\text{mL}$ ペニシリン、 $100 \text{U}/\text{mL}$ ストレプトマイシン (ナカライテスク社) および $55 \mu\text{M}$ β-ME (Gibco社)を添加したIMDM (Gibco社) (以下、basi

c c u l t u r e m e d i u mと略記する)で2回洗浄し、4 mLのb
a s i c c u l t u r e m e d i u mで懸濁し、最終濃度 $10\mu\text{g}/\text{mL}$
L r a b b i t p o l y c l o n a l a n t i h u m a n I g E
a n t i b o d y (D a k o社)を添加し、M A C S m i x t u b e
r o t a t o r (ミルテニーバイオテク社)を用いて回転させながら 37°C
、 $5\%\text{CO}_2$ インキュベーター内で1時間反応させた。

[0397] 反応後の細胞をSMで二回洗浄し、1ウェルあたり 5×10^4 個となるよう
に96ウェルプレートに播種し、 2000rpm で2分間の遠心分離を行っ
た。上清を除いた後、SMで希釈した $10000\mu\text{g}/\text{mL}$ I g G f r
o m h u m a n s e r u m (シグマアルドリッチ社)を $100\mu\text{L}/\text{w}$
e l lで添加して、 4°C 、30分間反応させ、E Z - L i n k S u l f o
- N H S - L C - B i o t i n , N o - W e i g h F o r m a t (P I E
R C E社)を用いて添付の説明書に従い、それぞれビオチン標識したヒト化
抗ヒトC R T H 2抗体L V O H V 1、抗ヒトC R T H 2抗体h u 1 9 A 2
v 5 2、c h 8 B 1、c h 3 C 1 2もしくはc h 3 1 A 5、または未標識の
市販の抗ヒトC R T H 2抗体B M 1 6 (s a n t a c r u z社)もしくは
3 0 1 1 0 8 (R & D社)を最終濃度 $10\mu\text{g}/\text{mL}$ で添加し、 4°C で1時
間の反応を行った。

[0398] なお、アイソタイプコントロール抗体として、B M 1 6に対してはP u r
i f i e d R a t I g G 2 a , κ I s o t y p e C t r l A n t
i b o d y (B i o L e g e n d社)を、3 0 1 1 0 8に対してはN e g a
t i v e C o n t r o l M o u s e I g G 2 a (D a k o社)を、そ
れ以外の抗体に対しては抗D N P I g G 1抗体を、それぞれ用いた。

[0399] 細胞を洗浄した後、ビオチン標識したL V O H V 1、h u 1 9 A 2 v 5
2、c h 8 B 1、c h 3 C 1 2またはc h 3 1 A 5を添加したウェルにはS
Mで希釈したs t r e p t a v i d i n、A l e x a F l u o r 6 4 7
c o n j u g a t e (M o l e c u l a r P r o b e s社)を、B M 1
6を添加したウェルにはSMで希釈したg o a t a n t i - R a t I g G

Alexa647 (Molecular Probes社) を、301108を添加したウェルにはSMで希釈したgoat anti-Mouse IgG Alexa647 (Molecular Probes社) を最終濃度10 μ g/mLで添加し、4℃、40分間の反応を行った。再び細胞を洗浄した後、細胞をSMで懸濁し、蛍光強度をフローサイトメトリー (BD バイオサイエンス社、FACS Cantoll) で解析した。

[0400] その結果、図14に示すように、hu19A2 v52がマスト細胞に対して反応性を示す一方で、LV0HV1を含むhu19A2 v52以外の抗体は反応性を示さなかった。

[0401] [実施例18]

分化誘導Th1に対する反応性解析

(1) PBMCからのナイーブT細胞の分離

健常人由来凍結PBMC (Allcells社) を、DNase I recombinant、RNase-free (Roche Diagnostics社) を50 μ L含有した20mLのPBMC培養培地で1回洗浄し、再びDNase I recombinant、RNase-free を50 μ L含有した20mLのPBMC培養培地に懸濁し、37℃、5%CO₂インキュベーター内で2時間放置した。

[0402] その後、実施例11に記載のMACS bufferで二回洗浄し、Naive CD4⁺ T Cell Isolation Kit II human (ミルテニーバイオテク社) を用いて、添付の説明書に従いナイーブCD4陽性T細胞をネガティブセレクション法により単離した。ナイーブCD4陽性T細胞の純度を高めるため、上記ネガティブセレクションを2回実施した。

[0403] (2) ナイーブCD4陽性T細胞からのTh1細胞の分化誘導

J Immunol, 2002. 169(5):p. 2498-2506記載の方法に準じて分離したナイーブCD4陽性T細胞を、10%FBS (Gibco社) および2mM グルタミン酸 (ナカライテスク社) を添加したRPMI1640 (Gibco

社) (以下、培養培地と略記する) で $1 \times 10^6 \text{ cells/mL}$ に懸濁し、刺激の工程として、抗CD3抗体OKT3 (Abcam社) を $1 \mu\text{g/mL}$ の濃度で固相化したプレートに播種し、最終濃度 $2 \mu\text{g/mL}$ 抗CD28抗体 (BDバイオサイエンス社)、 100 ng/mL recombinant human IL-12 (peprotech社)、 10 ng/mL recombinant human IL-2 (peprotech社)、および $5 \mu\text{g/mL}$ 抗IL-4抗体 (BDバイオサイエンス社) を添加し、 37°C 、 $5\% \text{ CO}_2$ インキュベーター内で4日間反応させた。

[0404] その後、細胞を回収し、遠心分離により上清を除去した後、培養培地で $1 \times 10^6 \text{ cells/mL}$ に懸濁し、増殖の工程として、最終濃度 100 ng/mL recombinant human IL-12 (peprotech社)、 10 ng/mL recombinant human IL-2 (peprotech社) および $5 \mu\text{g/mL}$ 抗IL-4抗体 (BDバイオサイエンス社) を添加し 37°C 、 $5\% \text{ CO}_2$ インキュベーター内で3日間反応させた。

[0405] 上記の刺激、増殖の工程を合計3回繰り返すことで、分化誘導Th1細胞を樹立した。

[0406] (3) 分化誘導Th1細胞からのサイトカイン産生解析

(2) の分化誘導Th1細胞培養液に、最終濃度 20 ng/mL の Phorbol 12-myristate 13-acetate (シグマアルドリッチ社)、 $1 \mu\text{g/mL}$ の Ionomycin calcium salt from Streptomyces (シグマアルドリッチ社) および $10 \mu\text{g/mL}$ の Brefeldin A from Penicillium brefeldianum (シグマアルドリッチ社) を添加し、 37°C 、 $5\% \text{ CO}_2$ インキュベーター内で6時間反応させた。その後、 $2 \times 10^5 \text{ cells/well}$ で96ウェルプレートに播種し、PBS (ナカライテスク社) で洗浄した。

[0407] その後、LIVE/DEAD Fixable Aqua Dead C

Cell Stain Kit, for 405nm excitation (Molecular Probes社)を用いて添付の説明書に従い死細胞を染色し、PBSで二回洗浄した後、上清を除き、Fixation Buffer (BDバイオサイエンス社)を用いて4℃、45分間反応させた。

[0408] 反応後、PBSで1回、1×Perm/Wash Buffer (BDバイオサイエンス社)で2回洗浄した後、最終濃度2.5μLのPE anti-human IFN-γ Antibody (BioLegend社)、5μLのAPC anti-human IL-4 Antibody (BioLegend社)、10μLのanti-IL-5 antibodies APC (ミルテニーバイオテク社)および20μLのAPC anti-human IL-13 Antibody (BioLegend社)を添加し、4℃で30分間反応させた。

[0409] その後、1×Perm/Wash Bufferで2回洗浄し、細胞をSMで懸濁し、蛍光強度をフローサイトメトリー (BDバイオサイエンス社、FACS Cantoll)で解析した。

[0410] その結果、分化誘導Th1は、IL-4/5/13が陰性であり、90%がIFNγ陽性のpopulationであることが確認され、Th1サイトカインを選択的に産生する細胞であることが確認できた。

[0411] (4) 分化誘導Th1細胞に対する抗ヒトCRTH2抗体の反応性解析

(2)において樹立された分化誘導Th1細胞をSMで洗浄した後、1ウェルあたり 5×10^4 cellsとなるように96ウェルプレートに播種し、2000rpmで2分間の遠心分離を行った。上清を除いた後、SMで希釈した10μg/mLラット抗ヒトCRTH2抗体Lym2、公知の抗ヒトCRTH2抗体 BM16 (santa cruz社)または301108 (R&D社)を添加し、4℃で1時間の反応を行った。

[0412] アイソタイプコントロール抗体として、Lym2に対してはRat IgG2b、kappa monoclonal [RTK4530]-BSA/

Azide free (abcam社) を、BM16に対してはPurified Rat IgG2a、 κ Isotype Ctrl Antibody (BioLegend社) を、301108に対してはNegative Control Mouse IgG2a (Dako社) を、それぞれ用いた。

[0413] 細胞を洗浄した後、Lym2抗体またはBM16を添加したウェルにはSMで希釈した $10\mu\text{g}/\text{mL}$ CELL LAB Mouse Anti-Rat Kappa (kappa light chain specific) FITC (ベクマンコールター社) を、301108を添加したウェルには、SMで希釈した $10\mu\text{g}/\text{mL}$ CELL LAB Goat Anti-Mouse IgG (γ chain specific) Fluorescein (FITC) Conjugate (ベクマンコールター社) を最終濃度 $10\mu\text{g}/\text{mL}$ でそれぞれ添加し、 4°C で1時間の反応を行った。再び細胞を洗浄した後、細胞をSMで懸濁し、蛍光強度をフローサイトメトリー (BDバイオサイエンス社、FACS Cantoll) で解析した。

[0414] その結果、図15に示すように、ラット抗ヒトCRTH2抗体Lym2および市販のラット抗ヒトCRTH2抗体BM16は分化誘導Th1には反応性を示さなかった。一方で、市販のマウス抗ヒトCRTH2抗体301108は分化誘導Th1に対して反応性を示した。

[0415] したがって、本発明の抗ヒトCRTH2抗体は、好酸球、好塩基球およびTh2細胞には反応するものの、Th1細胞には反応しないことが明らかになった。また、301108は、CRTH2を発現する細胞以外にも非特異的に反応性を示すことが明らかになった。

[0416] [実施例19]

ヒト好酸球の形態変化を指標にした、Lym2抗体のアンタゴニスト活性評価

実施例11と同様の方法により、ヒト末梢血から好酸球の分離を行った。

単離されたヒト好酸球をダルベッコリン酸緩衝生理食塩水（以下、D-PBS（－）と記す）（ナカライテスク社）で懸濁し、37℃、5%CO₂インキュベーター内で1時間30分反応させた。その後、10%FBS含有RPMI 1640を用いて2×10⁵ cells/wellで96ウェルプレートに播種した。その後、最終濃度10 μg/mLのアイソタイプコントロール抗体 [CELL LAB Rat IgG2b Isotype Control（ベックマンコールター社）]、もしくはLym2抗体、またはポジティブコントロールとして最終濃度10 μM CRTH2低分子拮抗薬OC000459（Cayman chemical社）を添加し、37℃、5%CO₂インキュベーター内で30分間反応させた。

[0417] 次に、0、1、1、10、100および1000 nM各最終濃度のDKPGD2（Cayman chemical社）を添加し、さらに37℃、5%CO₂インキュベーター内で60分間反応させた。反応後、氷冷PBSで2回洗浄し、Fixation Buffer（BDバイオサイエンス社）を200 μL/well添加し、よく懸濁した後、4℃で30分間放置した。その後、SMで二回洗浄し、フローサイトメトリー（BDバイオサイエンス社、FACS Cantor II）で好酸球の形態変化を解析した。

[0418] 好酸球の形態変化は、フローサイトメーターによる解析において細胞の大きさ、細胞の表面積または細胞径の指標であるForward Scatter Light（FSC）プロットの増加を指標に解析を行い、DKPGD2未処理の好酸球のFSCを基準として、高FSCのgateに検出される好酸球の割合（%）を算出した。

[0419] その結果、図16に示すように、ヒトCRTH2リガンドDKPGD2は、濃度依存的な好酸球の形態変化誘導をしたが、CRTH2低分子拮抗薬OC000459は、DKPGD2依存的な好酸球の形態変化を抑制した。これに対して、本発明の抗ヒトCRTH2抗体Lym2およびアイソタイプコントロール抗体は、いずれもDKPGD2依存的な好酸球の形態変化を抑制しなかった。即ち、本発明の抗ヒトCRTH2抗体はアンタゴニスト活性を

有しないことが示唆された。

[0420] [実施例 20]

ヒト好酸球の形態変化を指標にした、L y m 2 抗体のアゴニスト活性評価
上述実施例 19 と同様の実験系において、0、0.01、0.1、1 およ
び 10 μ g / mL 各最終濃度のラット抗ヒト C R T H 2 抗体 L y m 2 を加え
、1 時間反応させて、好酸球の形態変化を解析した。

[0421] その結果、図 17 に示すように、L y m 2 抗体は抗体濃度 0 – 10 μ g /
mL の範囲で処理を行っても好酸球の形態変化を起こさないことが示された
。即ち、本発明の抗ヒト C R T H 2 抗体はアゴニスト活性を有しないことが
示唆された。

[0422] [実施例 21]

ヒト好酸球の形態変化を指標にした、抗ヒト C R T H 2 抗体のアゴニスト活
性、アンタゴニスト活性、リガンドによるシグナルの増強活性の評価

実施例 11 と同様の方法により、ヒト末梢血から好酸球の分離を行った。
但し、血液に等量の注射用生理食塩水を添加する操作は行わず、直接 F i c
o l l – P a q u e P R E M I U M 1.084 (GEヘルスケア社) に
重層した。単離されたヒト好酸球を D – P B S (–) (ナカライテスク社)
で懸濁し、5 % C O₂ インキュベーター内で 37 °C、1 時間 30 分反応させた
。

[0423] その後、10 % F B S 含有 R P M I 1640 を用いて 0.7 × 10⁵ c e l
l s / w e l l で 96 ウェルプレートに播種した。その後、アイソタイプコ
ントロール抗体、ヒト化 L y m 2 抗体 L V O H V 1、実施例 9 で作成された
抗ヒト C R T H 2 抗体 h u 19 A 2 v 5 2、c h 8 B 1、c h 3 C 1 2 も
しくは c h 3 1 A 5、または市販の抗ヒト C R T H 2 抗体 B M 1 6 (s a n
t a c r u z 社) もしくは 301108 (R & D 社) を最終濃度 10 μ g
/ mL で添加し、37 °C、5 % C O₂ インキュベーター内で 30 分間反応させ
た。

[0424] なお、アイソタイプコントロール抗体として、B M 1 6 に対しては P u r

ified Rat IgG2a、 κ Isotype Ctrl Antibody (BioLegend社)、301108に対してはNegative Control Mouse IgG2a (Dako社)、その他の抗体に対しては抗DNP IgG1抗体を用いた。

[0425] 次に、100 nM最終濃度のDKPGD2 (Cayman chemical社) または10% FBS含有RPMI 1640を添加し、さらに37℃、5% CO₂インキュベーター内で60分間反応させた。反応後、氷冷PBSで2回洗浄し、Fixation Buffer (BDバイオサイエンス社) を100 μ L/well添加し、よく懸濁した後、4℃で30分間放置した。その後、SMで二回洗浄し、実施例19と同様の方法で好酸球の形態変化を解析した。

[0426] その結果、図18 (A) ~ (C) に示すように、リガンド非存在下における抗ヒトCRT H2抗体の処理による形態変化に関しては、hu19A2 v52において若干の形態変化の傾向が認められるものの、強い形態変化を起こす抗体は存在せず、いずれの抗体もアゴニスト活性を有しないことが示唆された。

[0427] また、DKPGD2処理条件下における抗ヒトCRT H2抗体の処理による形態変化に関しては、ch8B1、ch3C12、ch31A5の処理により、DKPGD2により誘発される形態変化が抑制されたことから、これらの抗体はアンタゴニスト活性を有することが示唆された。本結果は、先行特許文献 (国際公開2014/144865号) における知見と一致する。

[0428] 一方、hu19A2 v52およびBM16は、DKPGD2により誘発される形態変化を増強した。特にhu19A2 v52は、100 nMのDKPGD2により誘発される形態変化を約2倍まで増強することが明らかになった。

[0429] 上述の通り、hu19A2 v52およびBM16抗体はリガンド非存在下では形態変化を誘発しないことから、これらの抗体はCRT H2リガンドであるDKPGD2によるシグナルの増強活性を有することが示唆された。

[0430] 一方L V O H V 1 および3 0 1 1 0 8は、アイソタイプコントロール抗体と同様に、D K P G D 2非存在下、存在下のいずれの条件下においても、形態変化に影響を示さなかったことから、D K P G D 2によるシグナルの増強活性を有していないことが示唆された。

[0431] 本発明の抗ヒトC R T H 2抗体は、生理的シグナルを遮断または増強することがない点で好ましい。

[0432] [実施例22]

C R T H 2活性化状態の変遷に伴うコンフォメーション変化に対するC R T H 2モノクローナル抗体の反応性評価

(1) ホースラディッシュペルオキシダーゼ(H R P) 標識抗体の作製

P e r o x i d a s e L a b e l i n g K i t - N H 2 (D o j i n d o 社) を用いて、添付文書に従い、ヒト化L y m 2抗体L V O H V 1 およびh u 1 9 A 2 v 5 2にH R Pを直接標識した後、D - P B S (-) (ナカライテスク社) で1 m g / m L に希釈した。

[0433] (2) ヒトC R T H 2発現細胞の膜画分の調製

実施例1で樹立したヒトC R T H 2発現C H O / D G 4 4細胞を0. 0 2 % E D T A溶液(ナカライテスク社) に剥離し、4℃に冷却したD - P B S (-) を用いて細胞を洗浄した。M i n u t e P l a s m a M e m b r a n e P r o t e i n I s o l a t i o n K i t (I n v e n t B i o t e c h n o l o g i e s) を用いて、添付文書に従い膜画分を調製した。

[0434] (3) ヒトC R T H 2発現細胞の膜画分に対する抗ヒトC R T H 2抗体の反応性の評価

5 0 m M H E P E S (G i b c o 社)、5 m M M g C l ₂ (ナカライテスク社)、1 0 0 m M N a C l (ナカライテスク社) および1 m M E D T A (i n v i t o r g e n 社) を添加した5 0 m M T r i s - H C l b u f f e r p H 7. 4 (ナカライテスク社) (以下、E L I S A 反応液と記す) を、(2) にて調製した膜画分に加え、1 m g / m L になるように

調製し、プロテオセーブ1. 5 mLマイクロチューブ（住友ベークライト社）に、 $50\text{ }\mu\text{L}/\text{tube}$ で添加した。

[0435] 次に、 $500\text{ }\mu\text{M}$ GTP γ S（Roche Diagnostics社）または $500\text{ }\mu\text{M}$ GDP（Sigma-Aldrich社）を添加したELISA反応液を $50\text{ }\mu\text{L}/\text{tube}$ で添加し、 30°C にて、1時間インキュベートした。

[0436] 続いて、D-PBS（-）で $10\text{ mg}/\text{mL}$ に希釈したIgG from human serum（Sigma-Aldrich社）および $30\% \text{ w}/\text{v}$ BSA-PBS fatty acid free（Wako社）をそれぞれ $50\text{ }\mu\text{L}/\text{tube}$ で添加し、 30°C で、30分間インキュベートした。

[0437] 次に、（1）で作製した、HRP標識されたLV0HV1またはhu19A2 v52をELISA反応液により $5\text{ }\mu\text{g}/\text{mL}$ に希釈し、 $50\text{ }\mu\text{L}/\text{tube}$ で添加した後 30°C で1時間インキュベートすることにより、膜画分に対する抗体反応を行った。 16000 g にて 4°C で30分間遠心した後、上清を除去した。

[0438] その後、もともとGTP γ Sを添加していたチューブには $100\text{ }\mu\text{M}$ GTP γ Sを添加したELISA反応液、GDPを添加していたチューブには $100\text{ }\mu\text{M}$ GDPを添加したELISA反応液をそれぞれ $1\text{ mL}/\text{tube}$ で添加して、再度 16000 g にて 4°C で30分間遠心した後、上清を除去した。同様の操作を4回繰り返した後、D-PBS（-）を $100\text{ }\mu\text{L}/\text{tube}$ で添加し、膜画分を十分に懸濁した。

[0439] 懸濁液を96ウェルプレートに $30\text{ }\mu\text{L}/\text{well}$ で添加し、1-Step Ultra TMB-ELISA試薬（Thermo scientific社）を $100\text{ }\mu\text{L}/\text{well}$ で添加した。室温で10分間反応させた後、 $0.5\text{ mol}/\text{L}$ 硫酸（Wako社）を $100\text{ }\mu\text{L}/\text{well}$ で添加し、反応を停止させた。

[0440] SPECTRA max 340PCにより 480 nm における吸光度を

測定し、得られた結果をGraphpad Prism (ver. 6.05)を用いて解析した。また、Tukey's multiple comparisons testを適応し、有意差検定を併せて実施した。

[0441] その結果、図19に示すようにGDP処理時におけるhu19A2 v52の膜画分に対する反応性は、GTP γ S処理時に比べて有意に低下した ($p < 0.0001$)。一方、LV0HV1の反応性は、GTP γ SまたはGDP処理時においても、変化しなかった ($p > 0.1$)。

[0442] CRTH2はGPCRであり、GPCRは一般的に、活性化型コンフォメーションではGTPが結合し、不活性化型コンフォメーションではGDPが結合していることが知られている。上記結果の通り、本発明の抗ヒトCRTH2抗体は、GTPアナログのGTP γ SまたはGDPによる処理の有無に関わらずCRTH2への反応性が変化しなかったことから、CRTH2の活性化に伴うコンフォメーション変化に影響されず、一定の反応性を示す可能性が示唆された。

[0443] [実施例23]

アザミグリーン融合ヒトおよびカニクイザルCRTH2発現p h m A G 1 - M N L i n k e rベクターの作製

(1) アザミグリーン融合ヒトCRTH2発現p h m A G 1 - M N L i n k e rベクターの作製

実施例1の(1) - (i i) で作製されたヒトCRTH2遺伝子発現p A M o h に対し、プライマーhumanCRTH2azami-A (配列番号60) 及びhumanCRTH2azami-B (配列番号61) を用いて目的断片をPCRで増幅し、制限酵素BamHIおよびHindIIIを用いて、ベクターp h m A G 1 - M N L i n k e r (MBL社) と連結し、アザミグリーン融合ヒトCRTH2発現p h m A G 1 - M N L i n k e rベクターを構築した。

[0444] (2) アザミグリーン融合カニクイザルCRTH2発現p h m A G 1 - M N L i n k e rベクターの作製

全合成されたカニクイザルC R T H 2のc D N A（c D N A配列：配列番号62、アミノ酸配列：配列番号63）を用い、実施例1の（1）－（i i）と同様の方法で構築したカニクイザルC R T H 2発現p M o hベクターに対し、プライマーc y n o C R T H 2 a z a m i－A（配列番号64）およびc y n o C R T H 2 a z a m i－B（配列番号65）を用い、（1）と同様にしてアザミグリーン融合カニクイザルC R T H 2発現p h m A G 1－M N L i n k e rベクターを構築した。

[0445] [実施例24]

アザミグリーン融合ヒトC R T H 2発現C H O／D G 4 4細胞およびカニクイザルC R T H 2発現C H O／D G 4 4細胞の造成

実施例23で作製されたヒトおよびカニクイザルC R T H 2遺伝子発現p h m A G 1－M N L i n k e rベクターを制限酵素B s a I処理によって切断し、得られた直鎖状D N Aを精製し、滅菌水に溶解した。このD N Aをエレクトロポレーション法により、C H O／D G 4 4細胞に導入し、I M D M培養培地にて3日程度培養した。

[0446] その後、0.5mg/mL G 4 1 8（ナカライテスク社）を加えたI M D M選択培地で薬剤耐性細胞を選択した。選択した薬剤耐性細胞を、0.25% T r y p s i n－E D T A（ナカライテスク社）で剥離し、P B Sで洗浄した後、I M D M選択培地で懸濁した。その後、C e l l S o r t e r S H 8 0 0（S o n y社）を用いて、ヒトC R T H 2発現細胞およびカニクイザルC R T H 2発現細胞で同程度のアザミグリーン蛍光強度を示す細胞集団をそれぞれゲートし、1 c e l l／w e l lでソーティングを行い、拡大培養し、アザミグリーン融合ヒトC R T H 2発現C H O／D G 4 4細胞およびカニクイザルC R T H 2発現C H O／D G 4 4細胞をそれぞれ樹立した。

[0447] アザミグリーン融合ヒトC R T H 2発現C H O／D G 4 4細胞およびカニクイザルC R T H 2発現C H O／D G 4 4細胞について、上記と同様の方法で細胞調製を行い、フローサイトメトリー（B Dバイオサイエンス社、F A

CS Canto II) を用いてアザミグリーンの発現確認を行った。その結果、図 20 に示すように、それぞれの細胞のアザミグリーンの発現が同等であることを確認した。

[0448] [実施例 25]

ヒトまたはカニクイザル CRT H 2 に対するヒト化 L y m 2 抗体 L V O H V 1 の結合活性評価

実施例 24 で樹立されたアザミグリー融合ヒトまたはカニクイザル CRT H 2 発現 CHO / DG 4 4 細胞を、0.02% EDT A 溶液 (ナカライテスク社) で剥離し、PBS で洗浄した後、SM で懸濁した。次に、細胞数を 1 ウェルあたり 2×10^5 個となるように 96 ウェルプレートに播種し、3000、7500、1875、469、117、29、7 および 2 ng / mL 各最終濃度のヒト化 L y m 2 抗体 L V O H V 1 または抗 DNP I g G 1 抗体を添加し、4℃で40分間の反応を行った。

[0449] 細胞を SM で 3 回洗浄した後、SM で 10 μ g / mL の濃度に希釈した g o a t a n t i - H u m a n I g G a l e x a 6 4 7 (M o l e c u l a r P r o b e s 社) を 1 ウェルあたり 100 μ L 添加し、4℃で40分間の反応を行った。SM で細胞を洗浄した後、細胞を 100 μ L の SM で再懸濁し、蛍光強度をフローサイトメトリー (BD バイオサイエンス社、F A C S C a n t o I I) で測定した。

[0450] データは F l o w J o 7 . 6 5 (トミーデジタルバイオロジー社) によって解析した。その結果、図 21 に示す通り、L V O H V 1 が、ヒト CRT H 2 およびサル CRT H 2 に対し、ほぼ同等の結合性を示すことが明らかとなった。

産業上の利用可能性

[0451] 本発明によりヒト CRT H 2 の特徴的なエピトープを認識し、結合することで所望の活性を有する抗ヒト CRT H 2 抗体、該抗体断片、該抗体のアミノ酸配列をコードする DNA、該 DNA を含むベクター、該抗体を生産するハイブリドーマおよび抗体生産細胞、該抗体の製造方法、該抗体または抗体

断片を含む組成物、該抗体または抗体断片を用いるアレルギー性疾患、自己免疫疾患、好酸球増多や機能亢進を伴う疾患、T h 2細胞の増多や機能亢進を伴う疾患などの治療方法および診断方法、並びに該抗体または抗体断片を含む医薬および診断薬を提供することができる。

[0452] 本発明を特定の態様を用いて詳細に説明したが、本発明の意図と範囲を離れることなく様々な変更および変形が可能であることは、当業者にとって明らかである。なお、本出願は、2015年7月15日付けで出願された日本特許出願（特願2015-141633）に基づいており、その全体が引用により援用される。

配列表フリーテキスト

[0453] 配列番号3：人工配列の記載：humanCRTH2FLAG-Aの塩基配列

配列番号4：人工配列の記載：humanCRTH2FLAG-Bの塩基配列

配列番号5：人工配列の記載：FLAGタグ付ヒトCRTH2 cDNAの塩基配列

配列番号6：人工配列の記載：humanCRTH2FLAG-Cの塩基配列

配列番号7：人工配列の記載：humanCRTH2FLAG-Dの塩基配列

配列番号8：人工配列の記載：RatIgG2bH-Aの塩基配列

配列番号9：人工配列の記載：RatIgG2bH-Bの塩基配列

配列番号10：人工配列の記載：Ratk-Aの塩基配列

配列番号11：人工配列の記載：Ratk-Bの塩基配列

配列番号20：人工配列の記載：Lym2抗体 VH CDR1のアミノ酸配列

配列番号21：人工配列の記載：Lym2抗体 VH CDR2のアミノ酸配列

配列番号 22 : 人工配列の記載 : L y m 2 抗体 V H C D R 3 のアミノ酸配列

配列番号 23 : 人工配列の記載 : L y m 2 抗体 V L C D R 1 のアミノ酸配列

配列番号 24 : 人工配列の記載 : L y m 2 抗体 V L C D R 2 のアミノ酸配列

配列番号 25 : 人工配列の記載 : L y m 2 抗体 V L C D R 3 のアミノ酸配列

配列番号 26 : 人工配列の記載 : c h L y m 2 発現ベクター用 V H の合成 D N A

配列番号 27 : 人工配列の記載 : c h L y m 2 発現ベクター用 V L の合成 D N A

配列番号 28 : 人工配列の記載 : c h L y m 2 V H - A の塩基配列

配列番号 29 : 人工配列の記載 : c h L y m 2 V H - B の塩基配列

配列番号 30 : 人工配列の記載 : c h L y m 2 V H - C の塩基配列

配列番号 31 : 人工配列の記載 : c h L y m 2 V H - D の塩基配列

配列番号 32 : 人工配列の記載 : L V O の塩基配列

配列番号 33 : 人工配列の記載 : 合成コンストラクトのアミノ酸配列

配列番号 34 : 人工配列の記載 : L V 1 の塩基配列

配列番号 35 : 人工配列の記載 : 合成コンストラクトのアミノ酸配列

配列番号 36 : 人工配列の記載 : L V 2 a の塩基配列

配列番号 37 : 人工配列の記載 : 合成コンストラクトのアミノ酸配列

配列番号 38 : 人工配列の記載 : L V 2 b の塩基配列

配列番号 39 : 人工配列の記載 : 合成コンストラクトのアミノ酸配列

配列番号 40 : 人工配列の記載 : L V 2 c の塩基配列

配列番号 41 : 人工配列の記載 : 合成コンストラクトのアミノ酸配列

配列番号 42 : 人工配列の記載 : L V 3 a の塩基配列

配列番号 43 : 人工配列の記載 : 合成コンストラクトのアミノ酸配列

配列番号 44 : 人工配列の記載 : L V 3 b の塩基配列

配列番号 45 : 人工配列の記載 : 合成コンストラクトのアミノ酸配列

配列番号 46 : 人工配列の記載 : L V 4 の塩基配列

配列番号 47 : 人工配列の記載 : 合成コンストラクトのアミノ酸配列

配列番号 48 : 人工配列の記載 : H V 0 の塩基配列

配列番号 49 : 人工配列の記載 : 合成コンストラクトのアミノ酸配列

配列番号 50 : 人工配列の記載 : H V 1 の塩基配列

配列番号 51 : 人工配列の記載 : 合成コンストラクトのアミノ酸配列

配列番号 52 : 人工配列の記載 : H V 2 a の塩基配列

配列番号 53 : 人工配列の記載 : 合成コンストラクトのアミノ酸配列

配列番号 54 : 人工配列の記載 : H V 2 b の塩基配列

配列番号 55 : 人工配列の記載 : 合成コンストラクトのアミノ酸配列

配列番号 56 : 人工配列の記載 : H V 3 の塩基配列

配列番号 57 : 人工配列の記載 : 合成コンストラクトのアミノ酸配列

配列番号 58 : 人工配列の記載 : H V 4 の塩基配列

配列番号 59 : 人工配列の記載 : 合成コンストラクトのアミノ酸配列

配列番号 60 : 人工配列の記載 : h u m a n C R T H 2 a z a m i - A
の塩基配列

配列番号 61 : 人工配列の記載 : h u m a n C R T H 2 a z a m i - B
の塩基配列

配列番号 64 : 人工配列の記載 : c y n o C R T H 2 a z a m i - A の
塩基配列

配列番号 65 : 人工配列の記載 : c y n o C R T H 2 a z a m i - B の
塩基配列

請求の範囲

- [請求項1] 配列番号2で表されるヒトC R T H 2のアミノ酸配列の192番目のグリシンおよび194番目のアスパラギン酸の少なくとも一方を認識し、結合する抗体または該抗体断片。
- [請求項2] 抗体が、配列番号2で表されるヒトC R T H 2のアミノ酸配列の12番目のプロリン、13番目のイソロイシン、14番目のロイシン、15番目のグルタミン酸、177番目のアスパラギン酸、178番目のグリシン、179番目のアルギニン、180番目のイソロイシン、181番目のメチオニン、182番目のシステイン、183番目のチロシン、184番目のチロシン、185番目のアスパラギン、186番目のバリン、187番目のロイシン、188番目のロイシン、189番目のロイシン、195番目のアルギニン、196番目のアスパラギン酸、197番目のアラニン、および198番目のスレオニンからなる群から選ばれるアミノ酸残基の少なくとも1つを認識し、結合する抗体である請求項1に記載の抗体または該抗体断片。
- [請求項3] 抗体が、以下の(a)～(g)からなる群から選ばれるアミノ酸残基の少なくとも1つを認識する抗体である、請求項1または2に記載の抗体または該抗体断片。
- (a) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の12番目のプロリン、14番目のロイシンおよび15番目のグルタミン酸、
- (b) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の177番目のアスパラギン酸、178番目のグリシン、および179番目のアルギニン、
- (c) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の180番目のイソロイシンおよび181番目のメチオニン、
- (d) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の183番目のチロシン、184番目のチロシンおよび185番目のアスパラギン、

(e) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の187番目のロイシン、188番目のロイシンおよび189番目のロイシン、

(f) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の195番目のアルギニン、並びに

(g) 配列番号2で表されるヒトC R T H 2のアミノ酸配列の196番目のアスパラギン酸および198番目のスレオニン。

[請求項4]

抗体が、以下の(a)～(d)からなる群から選ばれるいずれか1つの抗体である、請求項1～3のいずれか1項に記載の抗体または該抗体断片。

(a) 抗体重鎖可変領域(以下、V Hと略記する)の相補性決定領域(以下、C D Rと略記する)1～3が、それぞれ配列番号20～22で表されるアミノ酸配列を含み、かつ抗体軽鎖可変領域(以下、V Lと略記する)のC D R 1～3が、それぞれ配列番号23～25で表されるアミノ酸配列を含む抗体、

(b) 配列番号49で表されるアミノ酸配列または配列番号49で表されるアミノ酸配列中の18番目のロイシンをメチオニンに、77番目のアスパラギンをセリンに、93番目のバリンをスレオニンに、および117番目のスレオニンをバリンに置換する改変から選ばれる少なくとも1つの改変が導入されたアミノ酸配列を含むV H並びに配列番号33で表されるアミノ酸配列または配列番号33で表されるアミノ酸配列中の2番目のイソロイシンをバリンに、4番目のメチオニンをロイシンに、15番目のプロリンをロイシンに、および85番目のアラニンをプロリンに置換する改変から選ばれる少なくとも1つの改変が導入されたアミノ酸配列を含むV Lを含む抗体、

(c) 配列番号49、51、53、55、57および59で表されるアミノ酸配列のいずれか1つを含むV H並びに配列番号33、35、37、39、41、43、45および47で表されるアミノ酸配列の

いずれか1つを含むV_Lを含む抗体、並びに

(d) 配列番号17で表されるアミノ酸配列を含むV_Hおよび配列番号19で表されるアミノ酸配列を含むV_Lを含む抗体。

[請求項5] 抗体が、下記(a)～(h)からなる群から選ばれる少なくとも1つの特徴を有する抗体である請求項1～4のいずれか1項に記載の抗体または該抗体断片。

(a) ヒトC_RT_H2のリガンド存在下でヒトC_RT_H2に対する反応性が低下しない、

(b) 中和活性を有しない、

(c) 抗体依存性細胞傷害(ADCC)活性を有する、

(d) マスト細胞およびT_h1細胞の少なくとも一方に反応しない、

(e) 好酸球、好塩基球、T_h2細胞および2型自然リンパ球(ILC2)から選ばれる少なくとも一つの細胞に反応する。

(f) アゴニスト活性を有しない、

(g) ヒトC_RT_H2のリガンドによるシグナルを増強しない、並びに

(h) 活性化状態または不活性化状態のヒトC_RT_H2に対する反応性が変化しない。

[請求項6] 抗体が、ヒトF_c領域を含む抗体である、請求項1～5のうちのいずれか1項に記載の抗体または該抗体断片。

[請求項7] 抗体が、モノクローナル抗体である請求項1～6のいずれか1項に記載の抗体または該抗体断片。

[請求項8] 抗体が、遺伝子組換え抗体である請求項1～7のいずれか1項に記載の抗体または該抗体断片。

[請求項9] 遺伝子組換え抗体が、ヒト型キメラ抗体、ヒト型CDR移植抗体およびヒト抗体から選ばれるいずれか1つの遺伝子組換え抗体である、請求項8に記載の遺伝子組換え抗体または該抗体断片。

[請求項10] 抗体が、サルC_RT_H2に結合する抗体である請求項1～9のいづ

れか1項に記載の抗体または該断片。

- [請求項11] Fab、Fab'、F(ab')₂、scFv、diabody、dsFvおよびCDRを含むペプチドから選ばれるいずれか1つの抗体断片である請求項1～10のいずれか1項に記載の該抗体断片。
- [請求項12] 請求項1～11のいずれか1項に記載の抗体または該抗体断片を産生するハイブリドーマ。
- [請求項13] 請求項1～11のいずれか1項に記載の抗体または該抗体断片をコードするDNA。
- [請求項14] 請求項13に記載のDNAを含有する組換え体ベクター。
- [請求項15] 請求項14に記載の組換え体ベクターを宿主細胞に導入して得られる形質転換株。
- [請求項16] 請求項12に記載のハイブリドーマまたは請求項15に記載の形質転換株を培地に培養し、培養物中に請求項1～11のいずれか1項に記載の抗体または該抗体断片を生産蓄積させ、該培養物から抗体または該抗体断片を採取することを特徴とする請求項1～11のいずれか1項に記載の抗体または該抗体断片の製造方法。
- [請求項17] 請求項1～11のいずれか1項に記載の抗体または該抗体断片を有効成分として含有する、ヒトCRTH2が関係する疾患の治療剤。
- [請求項18] 請求項1～11のいずれか1項に記載の抗体または該抗体断片を有効成分として含有する、ヒトCRTH2が関係する疾患の診断剤。
- [請求項19] CRTH2が関係する疾患がアレルギー性疾患、自己免疫疾患、好酸球増多および機能亢進の少なくとも一方を伴う疾患、Th2細胞の増多および機能亢進の少なくとも一方を伴う疾患、または2型自然リンパ球(ILC2)の増多および機能亢進の少なくとも一方を伴う疾患である請求項17または18に記載の剤。
- [請求項20] 請求項1～11のいずれか1項に記載の抗体または該抗体断片の有効量を投与することを含む、ヒトCRTH2が関係する疾患の治療方法。

- [請求項21] 請求項1～11のいずれか1項に記載の抗体または該抗体断片の有効量を投与することを含む、ヒトC R T H 2が関係する疾患の診断方法。
- [請求項22] ヒトC R T H 2が関係する疾患がアレルギー性疾患、自己免疫疾患、好酸球増多および機能亢進の少なくとも一方を伴う疾患、T h 2細胞の増多および機能亢進の少なくとも一方を伴う疾患、またはI L C 2の増多および機能亢進の少なくとも一方を伴う疾患である請求項20または21に記載の方法。
- [請求項23] ヒトC R T H 2が関係する疾患の治療および診断の少なくとも一方に使用するための、請求項1～11のいずれか1項に記載の抗体または該抗体断片。
- [請求項24] ヒトC R T H 2が関係する疾患がアレルギー性疾患、自己免疫疾患、好酸球増多および機能亢進の少なくとも一方を伴う疾患、T h 2細胞の増多および機能亢進の少なくとも一方を伴う疾患、またはI L C 2の増多および機能亢進の少なくとも一方を伴う疾患である請求項23に記載の抗体または該抗体断片。
- [請求項25] ヒトC R T H 2が関係する疾患の治療および診断剤の少なくとも一方の製造のための、請求項1～11のいずれか1項に記載の抗体または該抗体断片の使用。
- [請求項26] ヒトC R T H 2が関係する疾患がアレルギー性疾患、自己免疫疾患、好酸球増多および機能亢進の少なくとも一方を伴う疾患、T h 2細胞の増多および機能亢進の少なくとも一方を伴う疾患、またはI L C 2の増多および機能亢進の少なくとも一方を伴う疾患である請求項25に記載の使用。

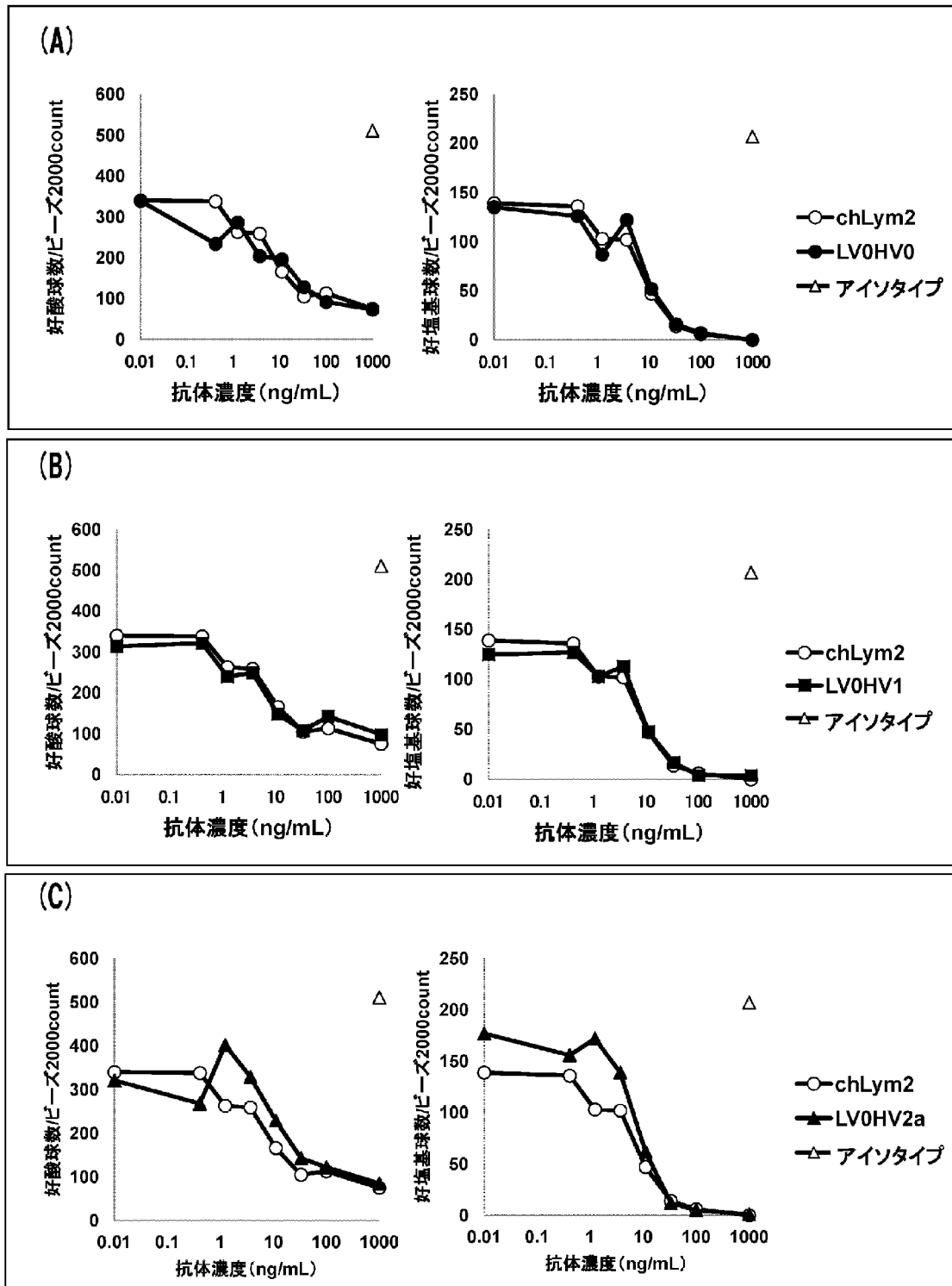
[図1]

	12345678901234567890123	4567890123456789	012345678901234	5678901
Lym2 VL	DVVLTQTPLSVTLGDAQASISC	RSSQSLEYSDBGYTYLE	WYLQKPGQSPQVLIY	GVSNRFS
LV0	DIVMTQTPLSLPVTLPGESASISC	CDR L1	WYLQKPGQSPQVLIY	CDR L2
LV1	DIVMTQTPLSLPVTLPGESASISC		WYLQKPGQSPQVLIY	
LV2a	DIVLTQTPLSLPVTLPGESASISC		WYLQKPGQSPQVLIY	
LV2b	DIVMTQTPLSLPVTLPGESASISC		WYLQKPGQSPQVLIY	
LV2c	DVVLTQTPLSLPVTLPGESASISC		WYLQKPGQSPQVLIY	
LV3a	DVVLTQTPLSLPVTLPGESASISC		WYLQKPGQSPQVLIY	
LV3b	DVVLTQTPLSLPVTLPGESASISC		WYLQKPGQSPQVLIY	
LV4	DVVLTQTPLSLPVTLPGESASISC		WYLQKPGQSPQVLIY	
	23456789012345678901234567890123	456789012	3456789012	
Lym2 VL	GVPDRFIGSGSGTDFTLKISRVEPEDLVGYVC	FQATHDPLT	FGSGTKLEIK	
LV0	GVPDRFIGSGSGTDFTLKISRVEAEDVGVYYC	CDR L3	FGQGTKLEIK	
LV1	GVPDRFIGSGSGTDFTLKISRVEAEDVGVYYC		FGQGTKLEIK	
LV2a	GVPDRFIGSGSGTDFTLKISRVEAEDVGVYYC		FGQGTKLEIK	
LV2b	GVPDRFIGSGSGTDFTLKISRVEAEDVGVYYC		FGQGTKLEIK	
LV2c	GVPDRFIGSGSGTDFTLKISRVEAEDVGVYYC		FGQGTKLEIK	
LV3a	GVPDRFIGSGSGTDFTLKISRVEAEDVGVYYC		FGQGTKLEIK	
LV3b	GVPDRFIGSGSGTDFTLKISRVEAEDVGVYYC		FGQGTKLEIK	
LV4	GVPDRFIGSGSGTDFTLKISRVEAEDVGVYYC		FGQGTKLEIK	

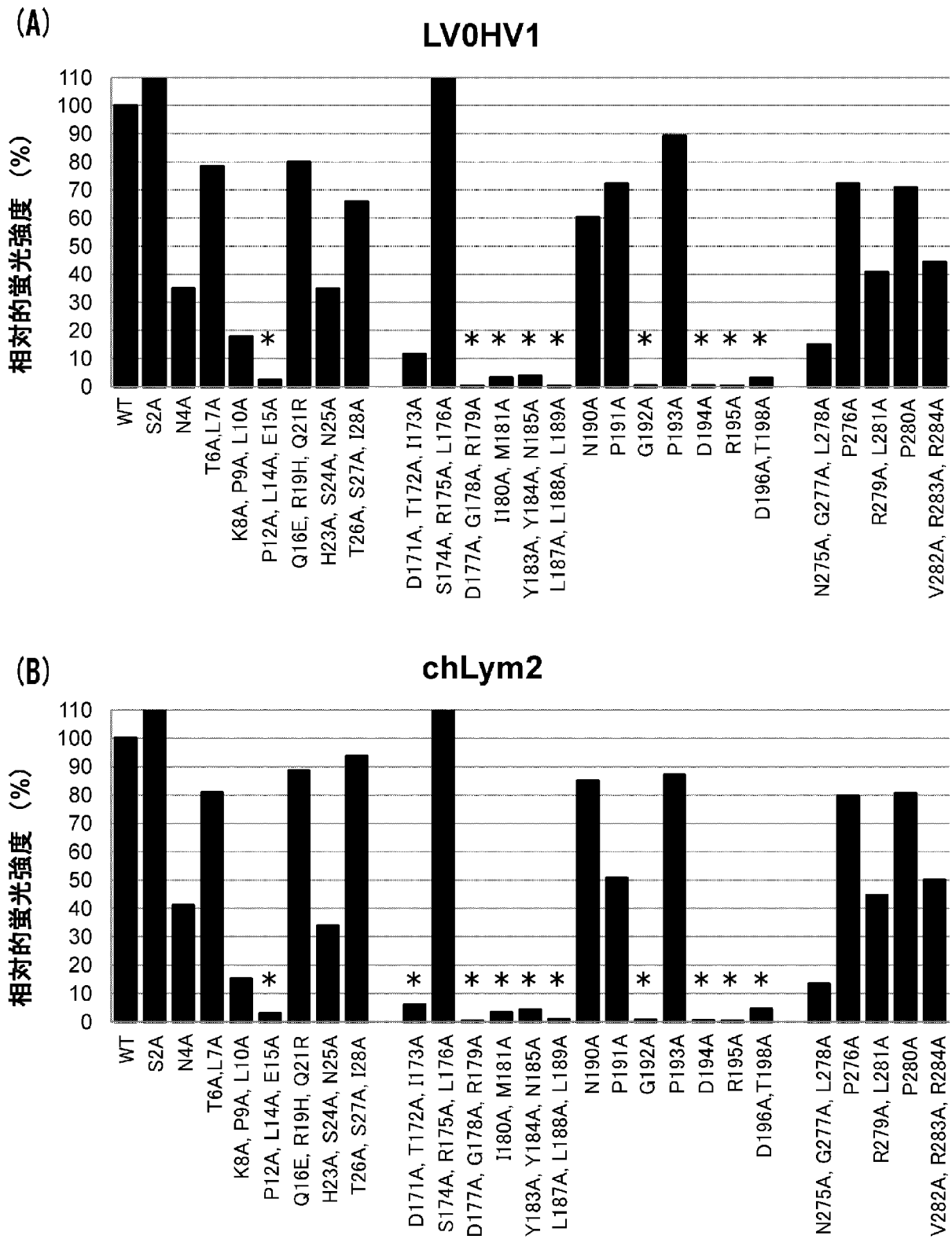
[図2]

	123456789012345678901234567890	12345	67890123456789	01234567890123456
Lym2 VH	EVQLVESGGGLVQPGRSMKLSCAASGFTFS	NYNMA	WVRQAPGKGLEWVA	TISYDGSSTYYRDSVKG
HV0	EVQLVESGGGVVQPGRSLRLSCAASGFTFS	CDR H1	WVRQAPGKGLEWVA	CDR H2
HV1	EVQLVESGGGVVQPGRSLRLSCAASGFTFS		WVRQAPGKGLEWVA	
HV2a	EVQLVESGGGVVQPGRSLRLSCAASGFTFS		WVRQAPGKGLEWVA	
HV2b	EVQLVESGGGVVQPGRSMRLSCAASGFTFS		WVRQAPGKGLEWVA	
HV3	EVQLVESGGGVVQPGRSMRLSCAASGFTFS		WVRQAPGKGLEWVA	
HV4	EVQLVESGGGVVQPGRSMRLSCAASGFTFS		WVRQAPGKGLEWVA	
	78901234567890123456789012345678	90123456789012	34567890123	
Lym2 VH	RFTISRDNASTLYLQMNSLRSEDATATYYCAR	HRGYYYSAGAGYFDY	WGQGVMTVSS	
HV0	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR	CDR H3	WGQGMVTVSS	
HV1	RFTISRDNAKNSLYLQMNSLRAEDTATYYCAR		WGQGMVTVSS	
HV2a	RFTISRDNAKNSLYLQMNSLRAEDTATYYCAR		WGQGVMTVSS	
HV2b	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR		WGQGVMTVSS	
HV3	RFTISRDNAKNSLYLQMNSLRAEDTATYYCAR		WGQGVMTVSS	
HV4	RFTISRDNAKSSLYLQMNSLRAEDTATYYCAR		WGQGVMTVSS	

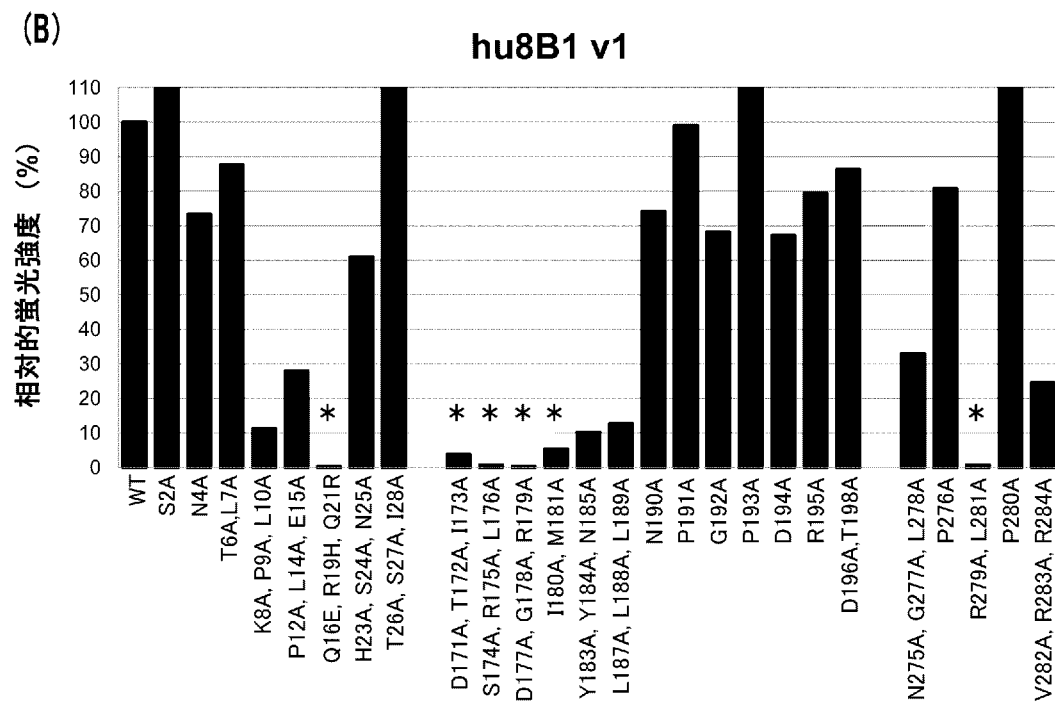
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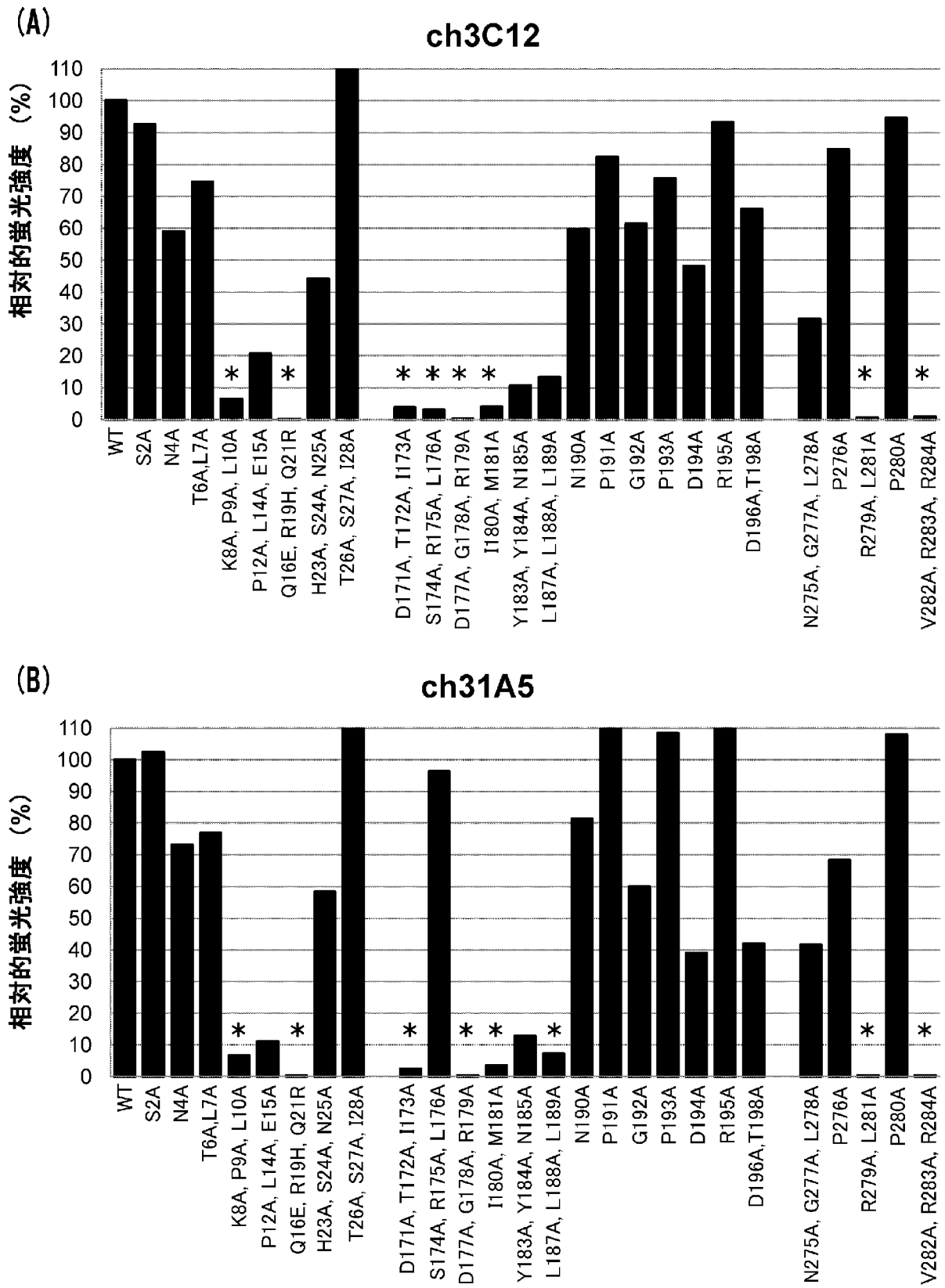
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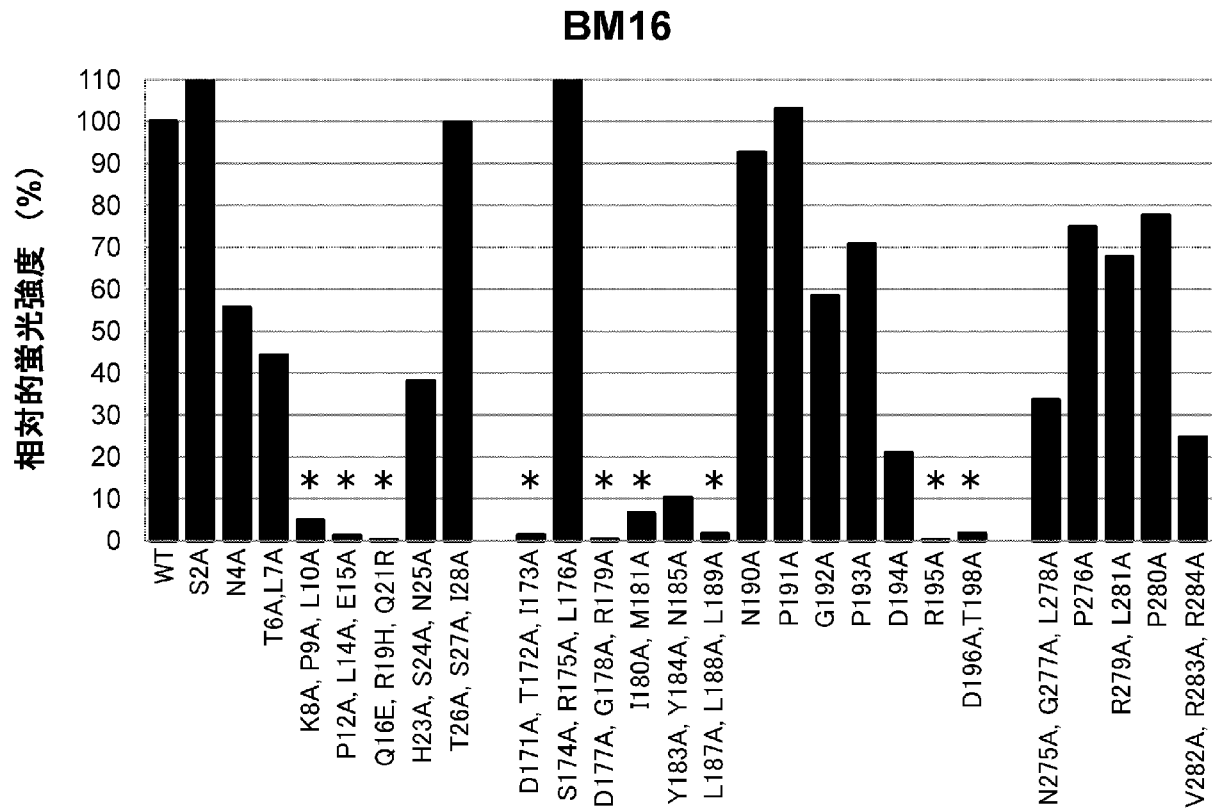
hu19A2 v52



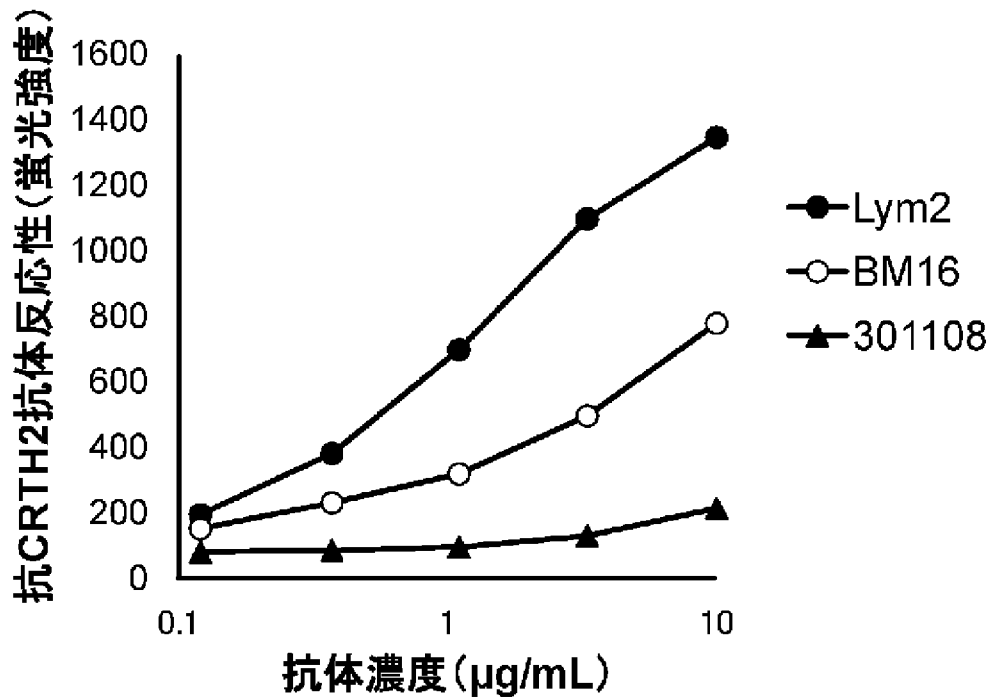
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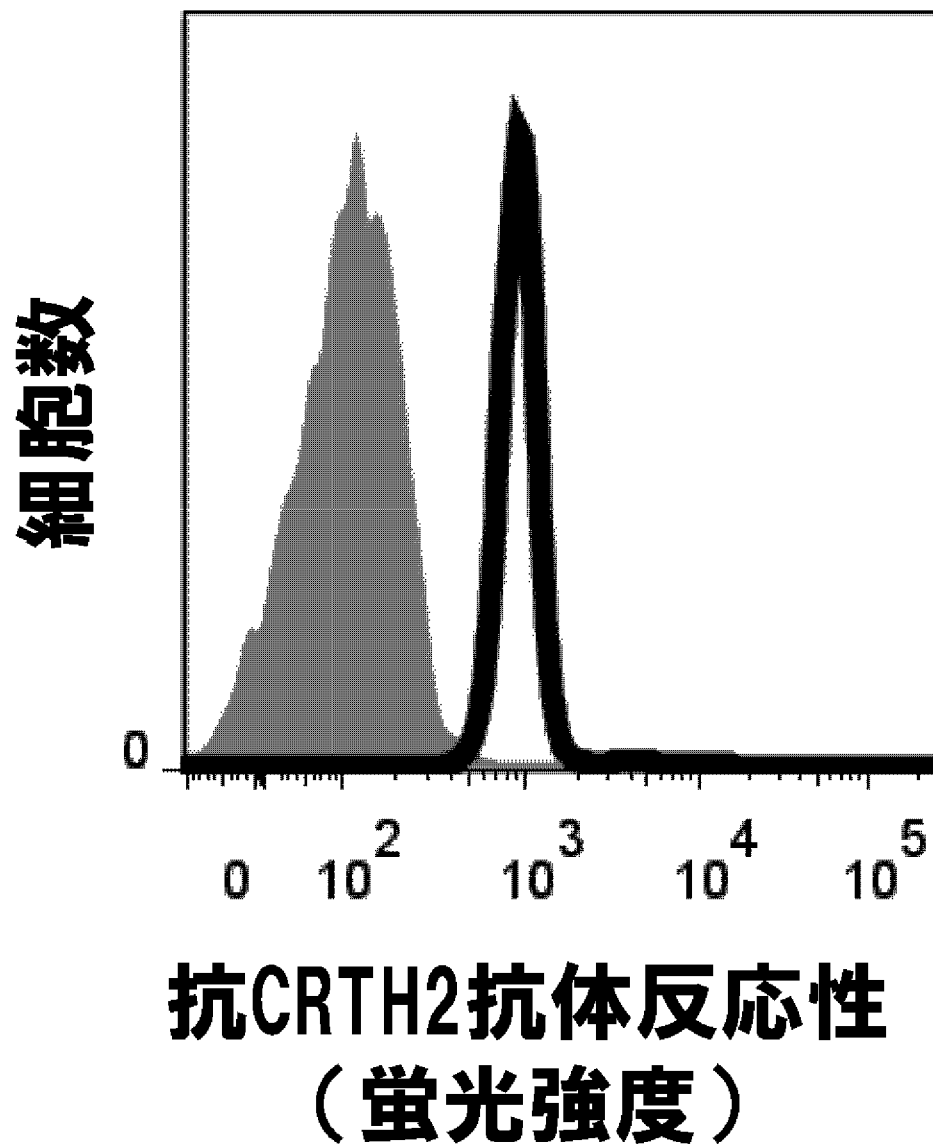
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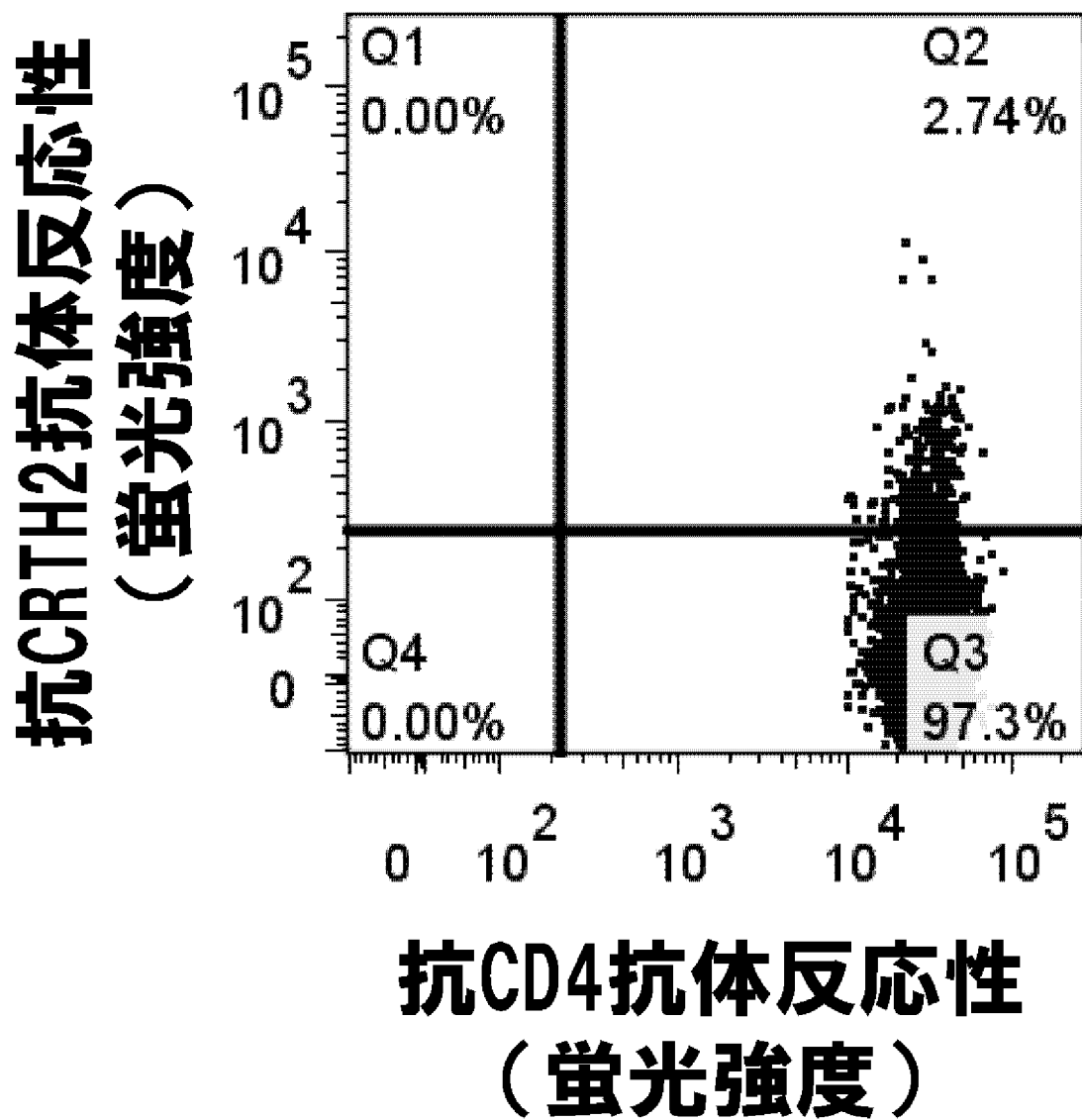
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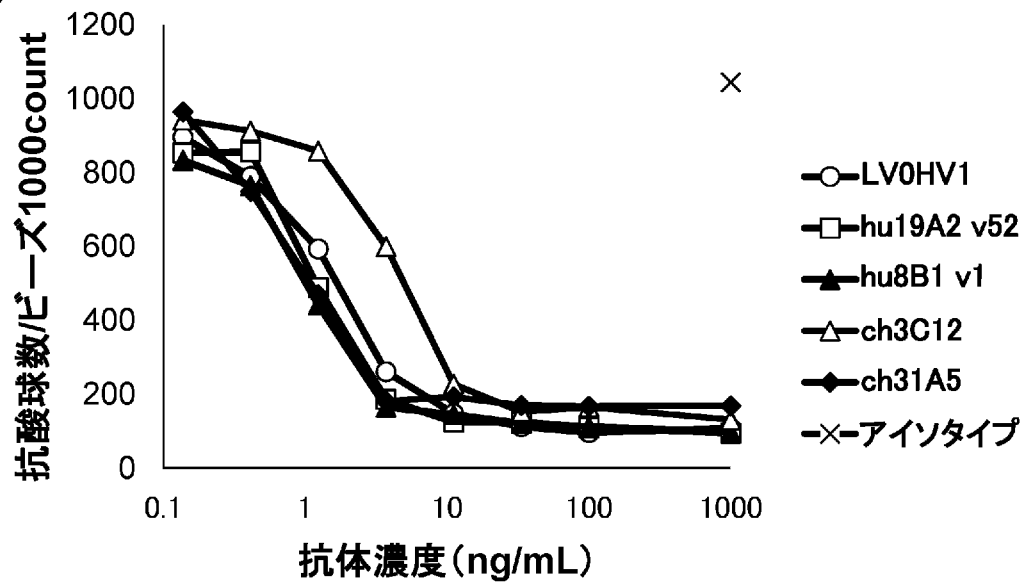


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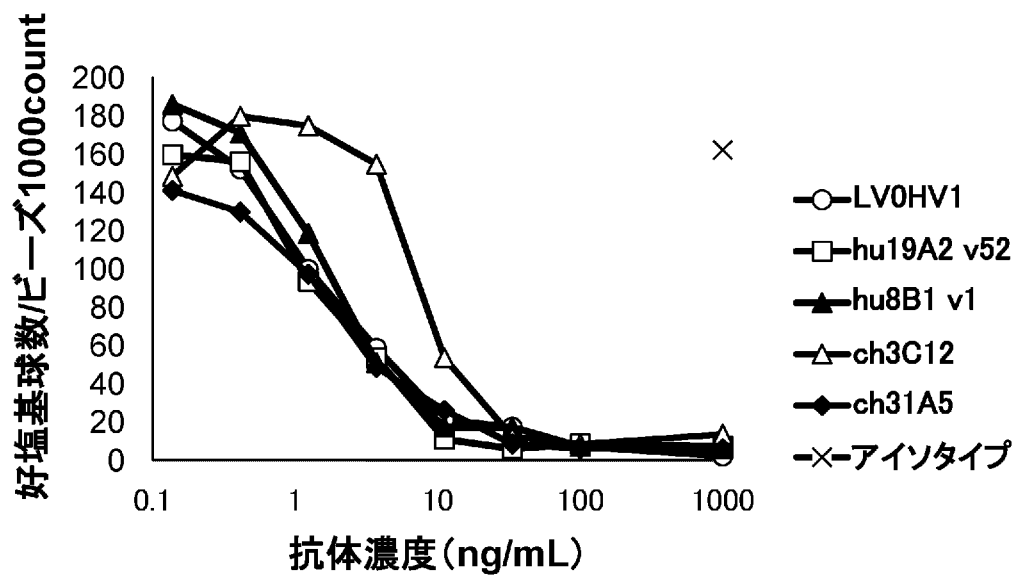


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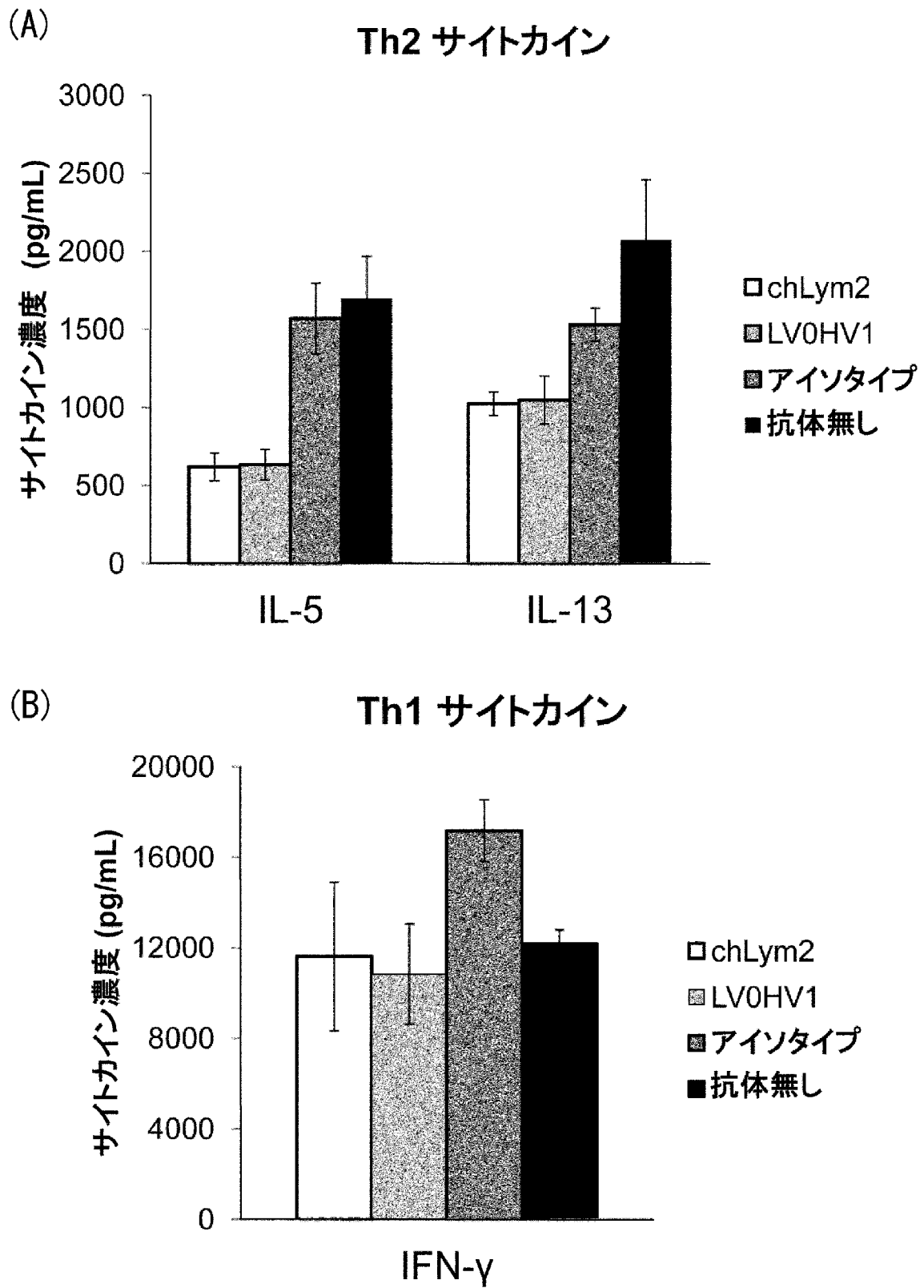
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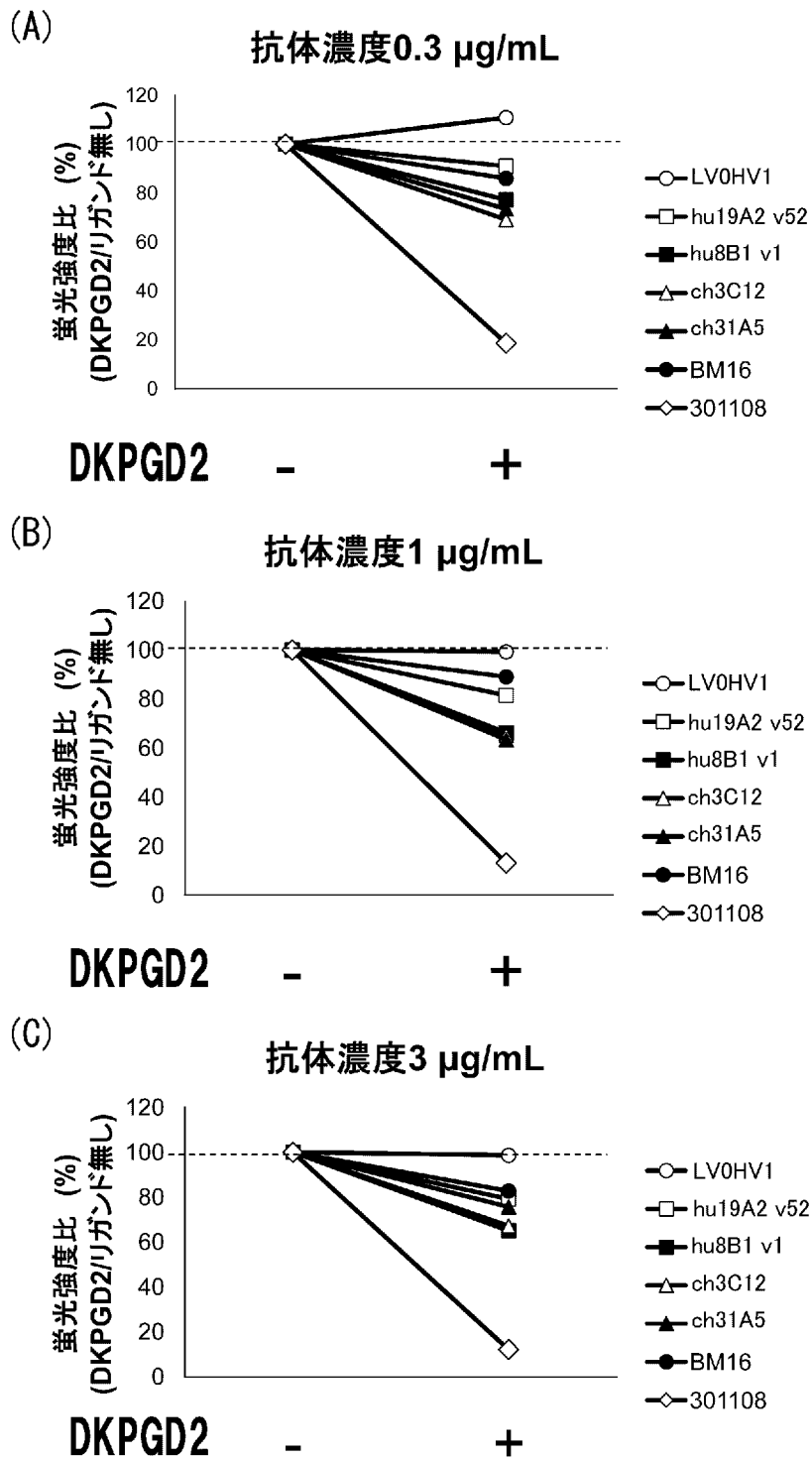
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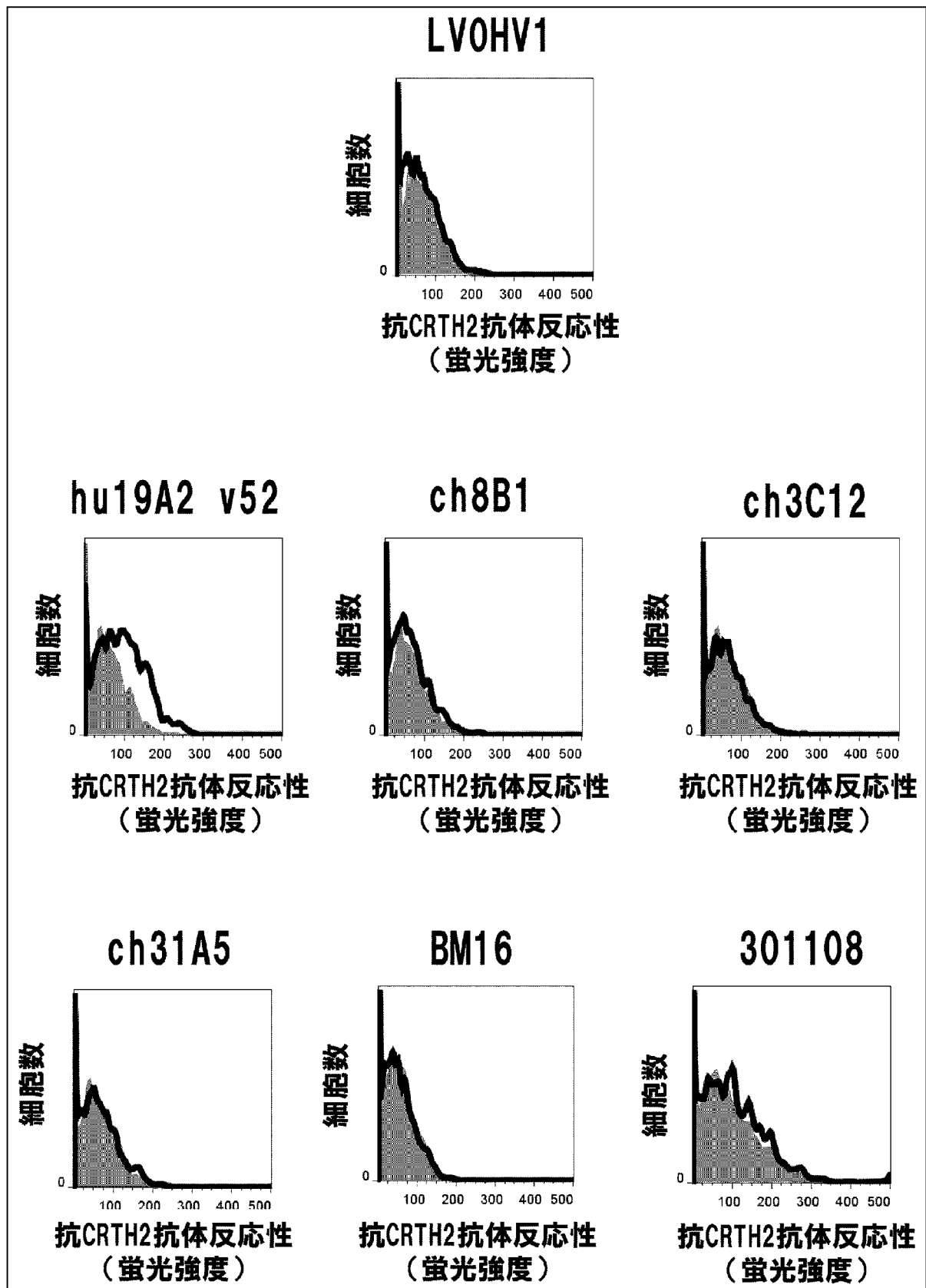
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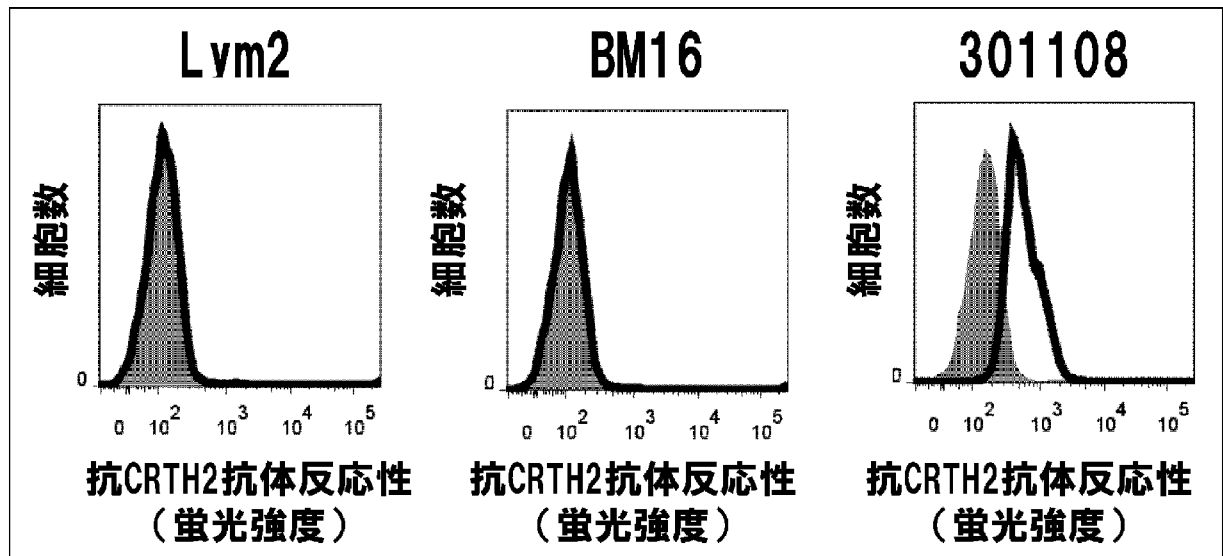
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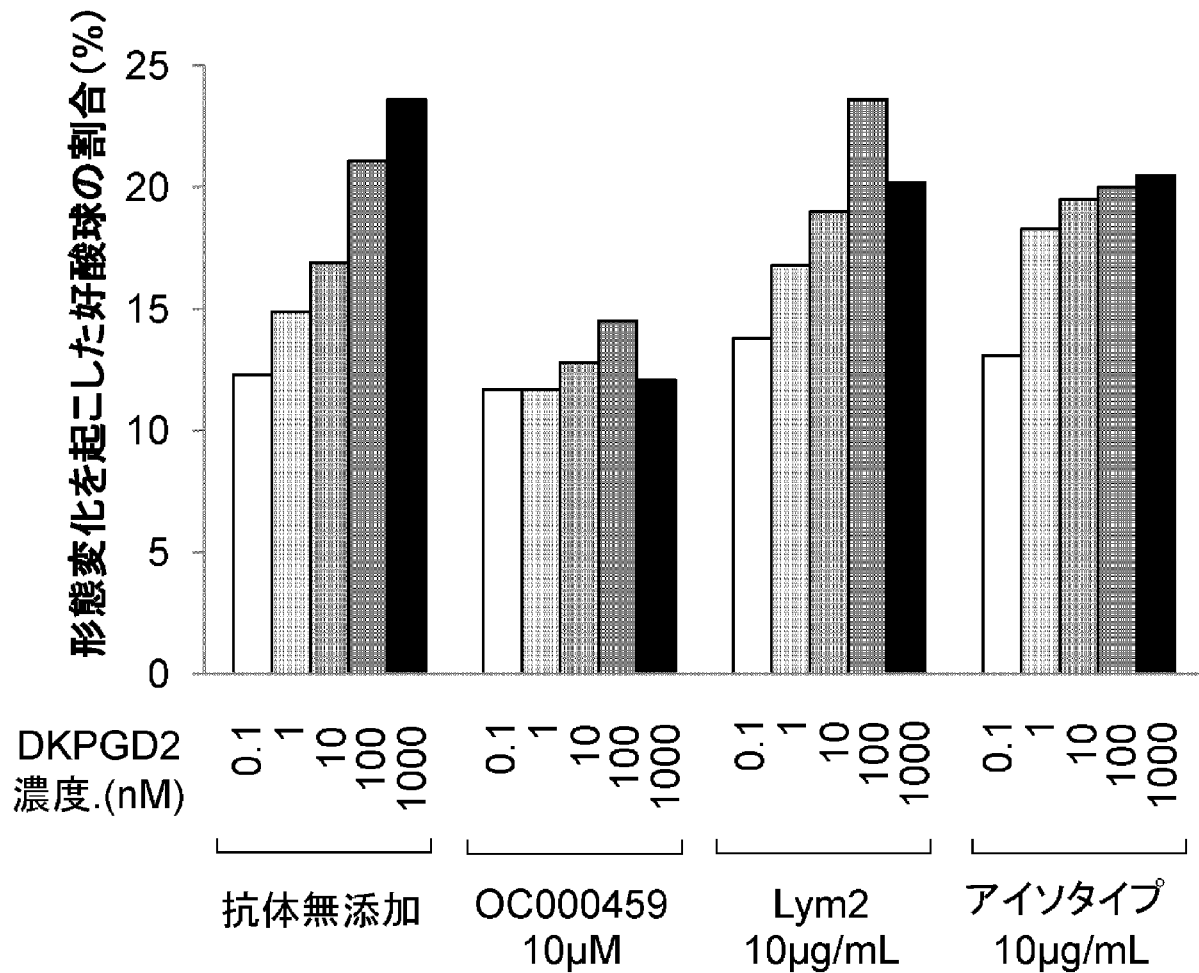
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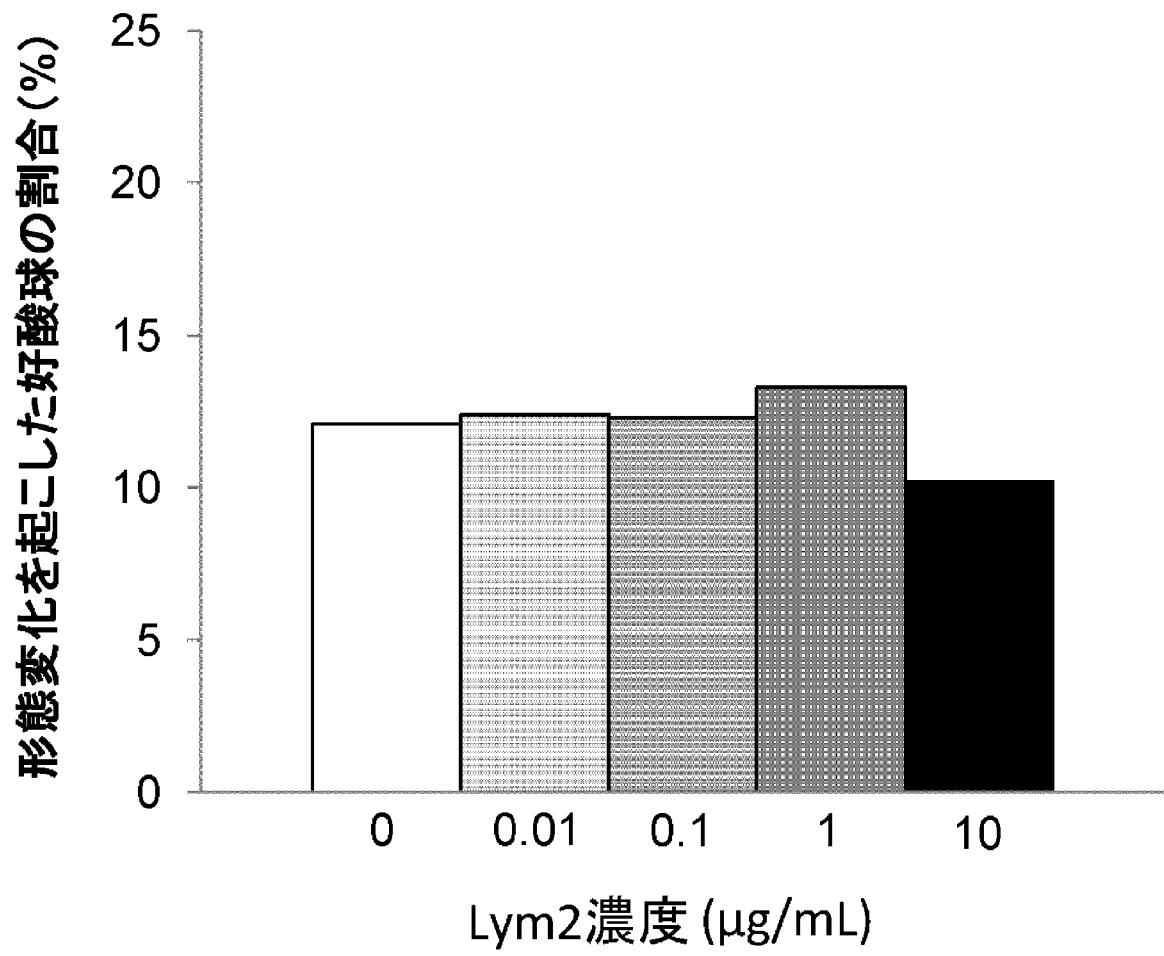
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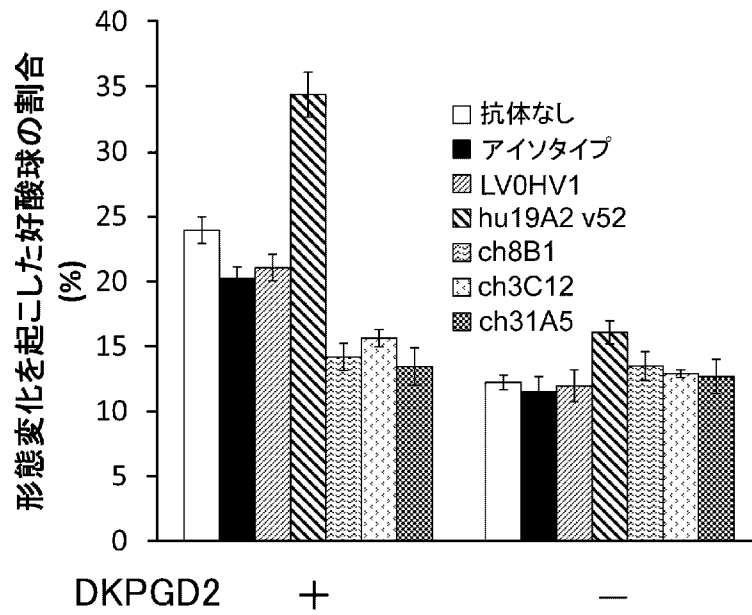


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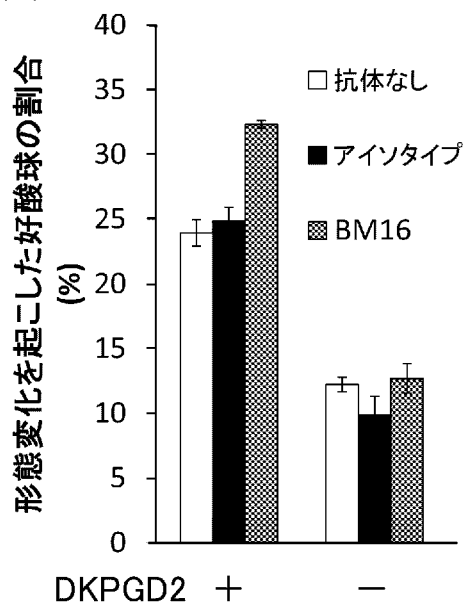


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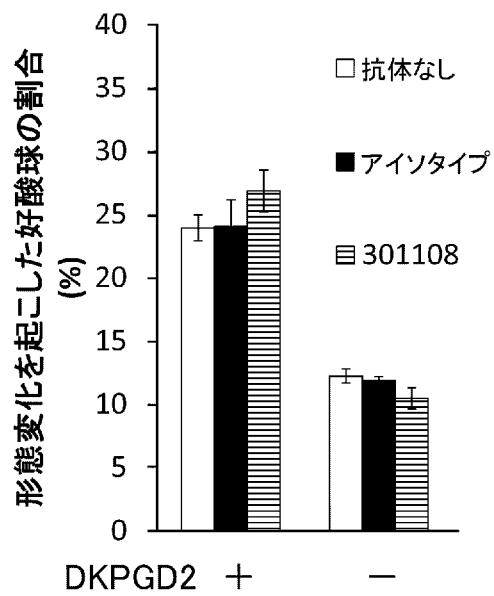
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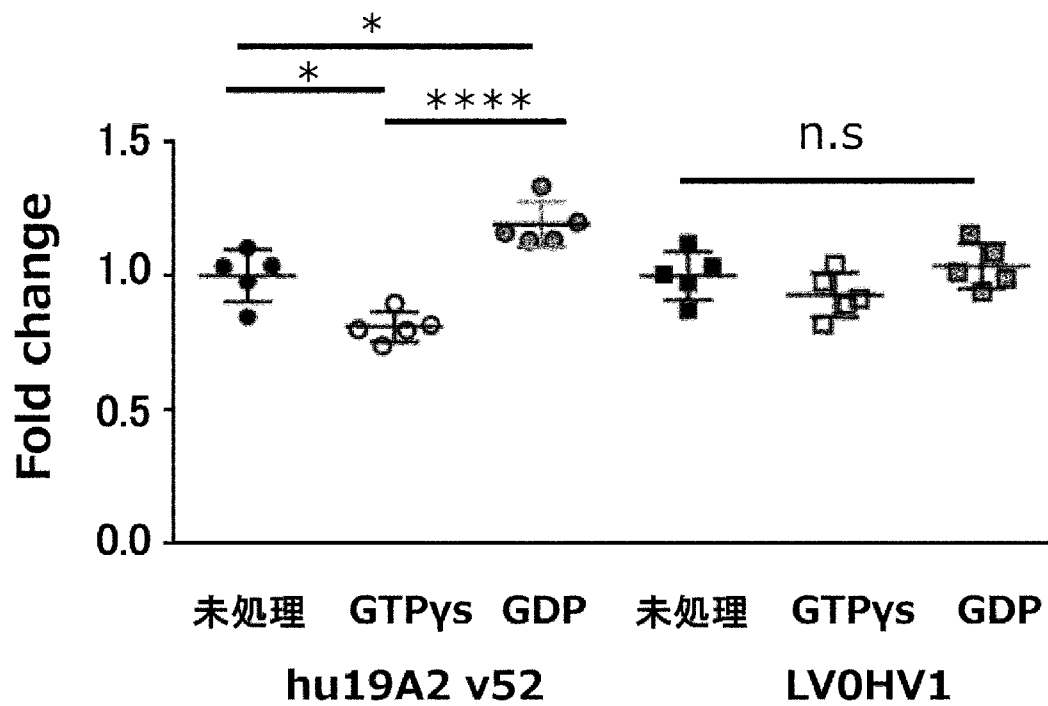
(B)



(C)

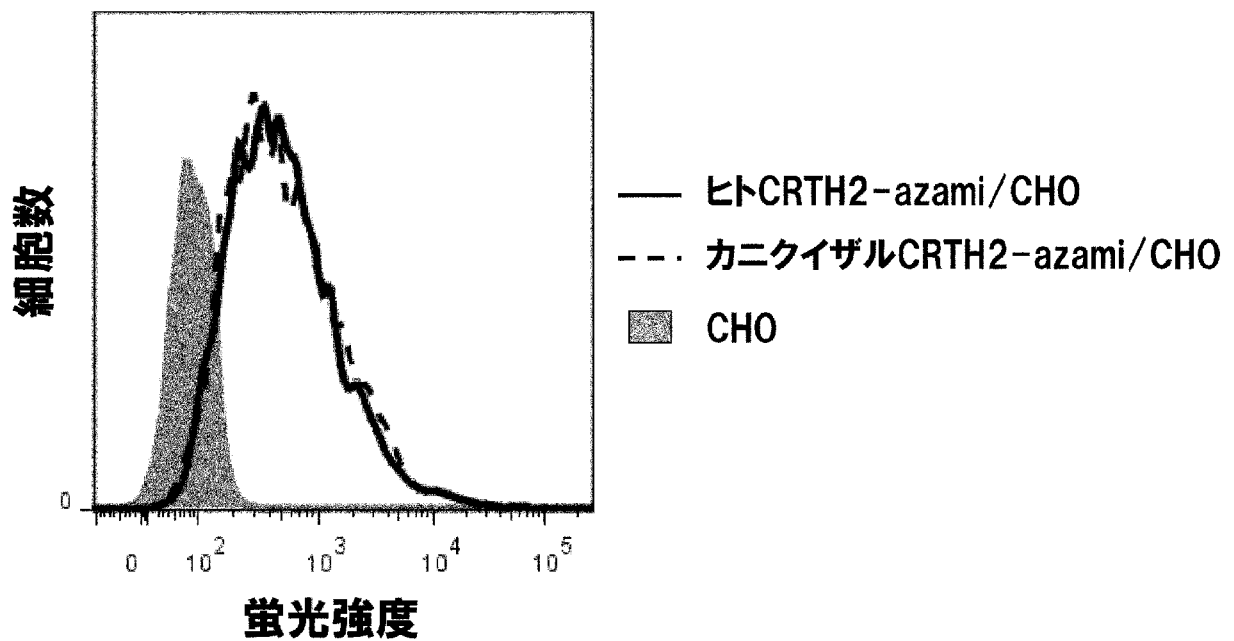


[図19]

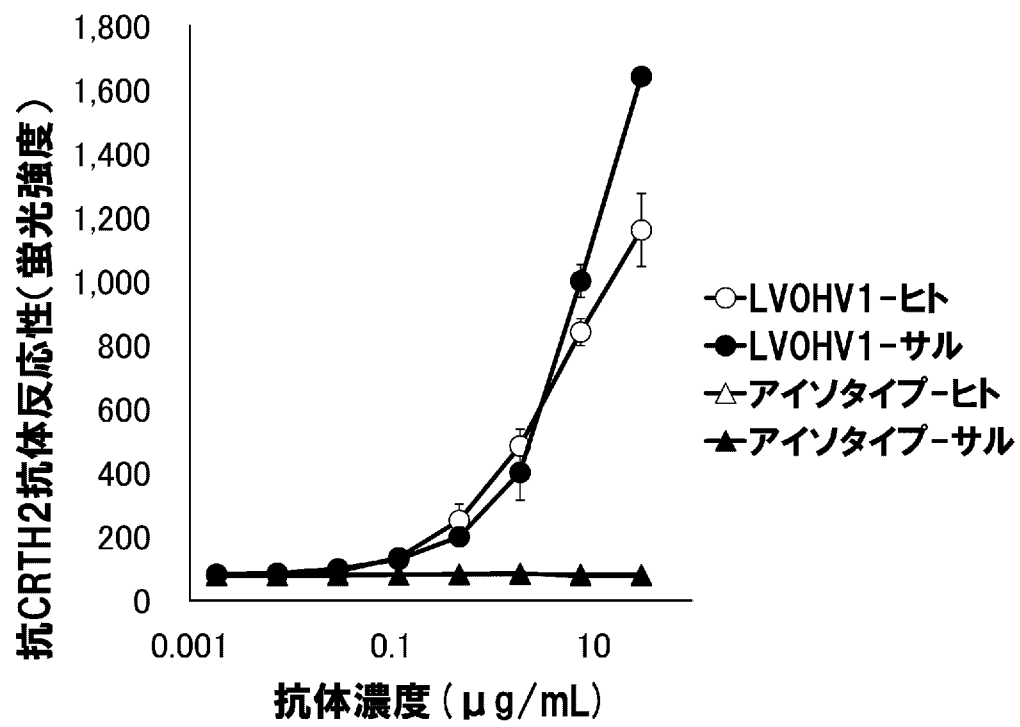


平均値±標準偏差 * $p < 0.05$, **** $p < 0.0001$, n.s $p > 0.1$

[図20]



[図21]



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2016/071027

A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K16/28, A61K39/395, A61P29/00, A61P37/02, A61P37/06, A61P37/08, C12N1/15, C12N1/19, C12N1/21, C12N5/10, C12N15/02, C12N15/09, C12P21/08, G01N33/53, G01N33/531

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2016
Kokai Jitsuyo Shinan Koho 1971-2016 Toroku Jitsuyo Shinan Koho 1994-2016

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

JSTPlus/JMEDPlus/JST7580(JDreamIII), CAlplus/MEDLINE/EMBASE/BIOSIS(STN), WPIDS/WPIX(STN), GenBank/EMBL/DDBJ/GeneSeq, UniProt/GeneSeq

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	WO 2014/144865 A2 (GENENTECH, INC.), 18 September 2014 (18.09.2014), claims; examples; paragraphs [0004], [0026] to [0028], [0199] to [0202] & JP 2016-517441 A & US 2014/0328849 A1 & EP 2970471 A2	1-3, 5-26 4
Y A	NAGATA, K., et al., Selective expression of a novel surface molecule by human Th2 cells in vivo, J. Immunol., 1999, vol.162, no.3, p.1278- 1286, ISSN 0022-1767, particularly, Materials and Methods, p.1280	1-3, 5-26 4



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

04 October 2016 (04.10.16)

Date of mailing of the international search report

11 October 2016 (11.10.16)

Name and mailing address of the ISA/

Japan Patent Office
3-4-3, Kasumigaseki, Chiyoda-ku,
Tokyo 100-8915, Japan

Authorized officer

Telephone No.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2016/071027

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	WO 1997/046677 A1 (BML, Inc.), 11 December 1997 (11.12.1997), claims; example 2 & US 6040426 A example 2 & EP 0851030 A1	1-3, 5-26 4
Y A	"CRTH-2/GPR44 Antibody FAB33381N", Novus Biologicals [online], 2015-06-26 Updated, [retrieved on 2016.10.04], Retrieved from the Internet: <URL: http://www.funakoshi.co.jp/ data/datasheet/NOV/FAB33381N.pdf >, entire text	1-3, 5-26 4
Y A	"Human CRTH-2 Antibody", R&D Systems, Inc. [online], 2015-03-13, [retrieved on 2016.10.04], Retrieved from the Internet: <URL: http://www. funakoshi.co.jp/data/datasheet/RSD/MAB3338.pdf >, entire text	1-3, 5-26 4
A	WO 2005/093092 A2 (BAYER HEALTHCARE AG), 06 October 2005 (06.10.2005), claims; examples (Family: none)	1-26

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2016/071027

Continuation of A. CLASSIFICATION OF SUBJECT MATTER

(International Patent Classification (IPC))

*C07K16/28(2006.01)i, A61K39/395(2006.01)i, A61P29/00(2006.01)i,
A61P37/02(2006.01)i, A61P37/06(2006.01)i, A61P37/08(2006.01)i,
C12N1/15(2006.01)i, C12N1/19(2006.01)i, C12N1/21(2006.01)i,
C12N5/10(2006.01)i, C12N15/02(2006.01)i, C12N15/09(2006.01)i,
C12P21/08(2006.01)i, G01N33/53(2006.01)i, G01N33/531(2006.01)i*

(According to International Patent Classification (IPC) or to both national
classification and IPC)

A. 発明の属する分野の分類（国際特許分類（I P C））

Int.Cl. 特別ページ参照

B. 調査を行った分野

調査を行った最小限資料（国際特許分類（I P C））

Int.Cl. C07K16/28, A61K39/395, A61P29/00, A61P37/02, A61P37/06, A61P37/08, C12N1/15, C12N1/19, C12N1/21, C12N5/10, C12N15/02, C12N15/09, C12P21/08, G01N33/53, G01N33/531

最小限資料以外の資料で調査を行った分野に含まれるもの

日本国実用新案公報	1 9 2 2 - 1 9 9 6 年
日本国公開実用新案公報	1 9 7 1 - 2 0 1 6 年
日本国実用新案登録公報	1 9 9 6 - 2 0 1 6 年
日本国登録実用新案公報	1 9 9 4 - 2 0 1 6 年

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

JSTPlus/JMEDPlus/JST7580 (JDreamIII)、Cplus/MEDLINE/EMBASE/BIOSIS (STN)、WPIDS/WPIX (STN)、GenBank/EMBL/DBJ/GeneSeq、UniProt/GeneSeq

C. 関連すると認められる文献

引用文献の カテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する 請求項の番号
Y A	W02014/144865 A2 (GENENTECH, INC) 2014. 09. 18, 特許請求の範囲, 実施例, 段落[0004], [0026]-[0028], [0199]-[0202] & JP 2016-517441 A & US 2014/0328849 A1 & EP 2970471 A2	1-3, 5-26 4

☒ C 欄の続きにも文献が列挙されている。☐ パテントファミリーに関する別紙を参照。

* 引用文献のカテゴリー

「A」特に関連のある文献ではなく、一般的技術水準を示すもの

「E」国際出願日前の出願または特許であるが、国際出願日以後に公表されたもの

「L」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献（理由を付す）

「O」口頭による開示、使用、展示等に言及する文献

「P」国際出願日前で、かつ優先権の主張の基礎となる出願

の日の後に公表された文献

「T」国際出願日又は優先日後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの

「X」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの

「Y」特に関連のある文献であって、当該文献と他の1以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの

「&」同一パテントファミリー文献

国際調査を完了した日

0 4 . 1 0 . 2 0 1 6

国際調査報告の発送日

1 1 . 1 0 . 2 0 1 6

国際調査機関の名称及びあて先

日本国特許庁（I S A / J P）

郵便番号 1 0 0 - 8 9 1 5

東京都千代田区霞が関三丁目4番3号

特許庁審査官（権限のある職員）

森井 文緒

電話番号 0 3 - 3 5 8 1 - 1 1 0 1 内線 3 4 4 8

4 B

3 7 6 5

C (続き) . 関連すると認められる文献		
引用文献の カテゴリ*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する 請求項の番号
Y A	NAGATA, K., et al., Selective expression of a novel surface molecule by human Th2 cells in vivo, J. Immunol., 1999, vol.162, no. 3, p.1278-1286, ISSN 0022-1767, 特に Materials and Methods, p. 1280	1-3, 5-26 4
Y A	WO 1997/046677 A1 (株式会社 ビー・エム・エル) 1997. 12. 11, 請求の範囲, 実施例 2 & US 6040426 A, 実施例 2 & EP 0851030 A1	1-3, 5-26 4
Y A	"CRTH-2/GPR44 Antibody FAB33381N", Novus Biologicals [online], 2015-06-26 Updated, [retrieved on 2016.10.04], Retrieved from the Internet: <URL: http://www.funakoshi.co.jp/data/datasheet/NOV/FAB33381N.pdf >, 全文	1-3, 5-26 4
Y A	"Human CRTH-2 Antibody", R&D Systems, Inc. [online], 2015-03-13, [retrieved on 2016.10.04], Retrieved from the Internet: <URL: http://www.funakoshi.co.jp/data/datasheet/RSD/MAB3338.pdf >, 全文	1-3, 5-26 4
A	WO 2005/093092 A2 (BAYER HEALTHCARE AG) 2005. 10. 06, 特許請求の範囲, 実施例 (ファミリーなし)	1-26

発明の属する分野の分類

C07K16/28(2006.01)i, A61K39/395(2006.01)i, A61P29/00(2006.01)i, A61P37/02(2006.01)i, A61P37/06(2006.01)i, A61P37/08(2006.01)i, C12N1/15(2006.01)i, C12N1/19(2006.01)i, C12N1/21(2006.01)i, C12N5/10(2006.01)i, C12N15/02(2006.01)i, C12N15/09(2006.01)i, C12P21/08(2006.01)i, G01N33/53(2006.01)i, G01N33/531(2006.01)i



(11)

EP 3 323 832 A1

(12)

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published in accordance with Art. 153(4) EPC

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A61P 29/00 ^(2006.01)	A61P 37/02 ^(2006.01)
A61P 37/06 ^(2006.01)	A61P 37/08 ^(2006.01)
C12N 1/15 ^(2006.01)	C12N 1/19 ^(2006.01)
C12N 1/21 ^(2006.01)	C12N 5/10 ^(2006.01)
C12N 15/02 ^(2006.01)	C12N 15/09 ^(2006.01)
C12P 21/08 ^(2006.01)	G01N 33/53 ^(2006.01)
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Designated Validation States:

MA MD

(30) Priority: **15.07.2015 JP 2015141633**

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Tokyo 100-8185 (JP)

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(74) Representative: **Hoffmann Eitle**

Patent- und Rechtsanwälte PartmbB
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(54) **ANTIBODY WHICH SPECIFICALLY BINDS TO HUMAN CRTH2**

(57) The present invention pertains to: an anti-human CRTH2 antibody which specifically recognizes and binds to human CRTH2; a fragment of the antibody; DNA which encodes the amino acid sequence of the antibody; a vector including the DNA; an antibody-producing cell and a hybridoma which produce the antibody; a production method for the antibody; a composition which includes the antibody or antibody fragment; a treatment

method and a diagnostic method which use the antibody or antibody fragment for diseases, such as allergenic diseases, autoimmune diseases, diseases associated with eosinophilia and hyperfunction, and diseases associated with an increase in and/or hyperfunction of Th2 cells; and a medicine and a diagnostic agent which include the antibody or antibody fragment.

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Description

Technical Field

5 **[0001]** The present invention relates to an anti-human CRTH2 antibody which specifically recognizes and binds to human CRTH2; the antibody fragment thereof; DNA which encodes the amino acid sequence of the antibody; a vector which comprises the DNA; hybridomas and antibody producing cells which produce the antibody; a method of producing the antibody; a composition which comprises the antibody or the antibody fragment thereof; a treatment method and a
 10 diagnostic method of an allergic disease, an autoimmune disease, a disease accompanied by increase or hyperergasia of eosinophilis, and a disease accompanied by increase or hyperergasia of Th2 cells using the antibody or the antibody fragment thereof; and a medicine and a diagnostic agent which comprise the antibody or the antibody fragment thereof.

Background Art

15 **[0002]** Human CRTH2 (chemoattractant receptor-homologous molecule on Th2 cells) is a seven transmembrane type G protein-coupled receptor (hereinafter, referred to as GPCR) that is also known as the aliases of GPR44, CD294, DP2, and the like. Further, it is known that human CRTH2 is one of receptors of prostaglandin D2 (hereinafter, referred to as PGD2) (Non-Patent Document 1). It is disclosed that CRTH2 was cloned as human Th2-specific protein in 1996 and referred to as B19 (Patent Document 1).

20 **[0003]** It is known that CRTH2 binds to PGD2 and PGD2 metabolites typified by 13, 14-dihydro-15-keto prostaglandin D2 (hereinafter, referred to as DKPGD2) which are ligands, transmits a G α i protein-mediated signal into cells, and accordingly, is involved in migration and activation of CRTH2-expressing cells (Non-Patent Document 1).

[0004] Human CRTH2 is recognized to be expressed on Th2 cells, eosinophils, basophils, type 2 innate lymphoid cells (hereinafter, referred to as ILC2), or the like (Non-Patent Documents 1 and 2). It is reported that CRTH2 is a surface
 25 marker specifically expressed on Th2 cytokine-producing cells (Non-Patent Document 3).

[0005] Moreover, ILC2 is a novel cell population involved in allergic responses identified in humans in 2011 and CRTH2 is exemplified as a specific surface marker defining the cells (Non-Patent Document 2). Further, it is reported that CRTH2 is expressed on non-classical monocyte or Th2/Th17 cells (Non-Patent Documents 4 and 5).

30 **[0006]** In allergic diseases including asthma, it is known that CRTH2-expressing cells contribute to the pathology. It is reported that CRTH2-positive T-cells are recognized at a higher frequency in cells in bronchoalveolar lavage fluid of asthma patients than in cells of the healthy people (Non-Patent Document 6) and CRTH2-positive T-cells increase in correlation with the severity of atopic dermatitis (Non-Patent Document 7).

[0007] Since eosinophils contain granule protein having cellular cytotoxicity and deposition of the protein is found in respiratory tract tissues of chronic bronchial asthma patients or lesions of atopic dermatitis patients, it is considered that eosinophils play an important role in pathogenesis of allergic diseases such as chronic bronchial asthma or atopic
 35 dermatitis (Non-Patent Documents 8 and 9).

[0008] Basophils are involved in induction of an allergic reaction by storing inflammatory molecules such as histamine and leukotriene in cells and releasing them by crosslinking an Fc ϵ receptor or an Fc γ receptor that is expressed on the cell surface to release the molecules (Non-Patent Document 10).

40 **[0009]** ILC2 is a cell present in a local area such as respiratory tract mucosa or skin. It has characteristics of producing a large amount of Th2 cytokines in response to cytokines such as interleukin (hereinafter, referred to as IL)-25 and IL-33 produced due to tissue injury, and is considered to be involved in pathogenesis of allergic diseases (Non-Patent Document 11).

[0010] As monoclonal antibody against CRTH2, 301108 (R&D) is commercially available. Further, BM16 is also known (Patent Document 2). These are rodent antibodies and have not been developed as medicine.

[0011] Moreover, it is also disclosed that a recombinant chimeric antibody and a humanized antibody regarding clone 19A2 remove CRTH2-expressing cells by effector activity and a humanized antibody regarding clone 8B1 and mouse antibodies regarding clones 3C12 and 31A5 have antagonist activity against CRTH2.

50 **[0012]** Further, it is disclosed that an antibody regarding clone 19A2 has reactivity to human mast cells (Patent Document 3).

Related Art

Patent Document

55 **[0013]**

[Patent Document 1] Japanese Patent No. 3144805

[Patent Document 2] Pamphlet of International Publication No. 97/46677
 [Patent Document 3] Pamphlet of International Publication No. 2014/144865

Non-Patent Document

[0014]

[Non-Patent Document 1] The Journal of Experimental Medicine, 2001. 193(2): p. 255 to 261.
 [Non-Patent Document 2] Nature Immunology, 2011. 12(11): p. 1055 to 1062.
 [Non-Patent Document 3] European Journal of Immunology, 2000. 30(10): p. 2972 to 2979.
 [Non-Patent Document 4] Blood, 2011. 118(5): e16 to 31
 [Non-Patent Document 5] Journal of Allergy and Clinical Immunology, 2014. 134(5): p. 1175 to 1186. e7.
 [Non-Patent Document 6] Clinical & Experimental Immunology, 2010. 161(1): p. 34 to 40.
 [Non-Patent Document 7] Journal of Investigative Dermatology, 2002. 119(3): p. 609 to 616.
 [Non-Patent Document 8] Advances in Immunology, 1986. 39: p. 177 to 253.
 [Non-Patent Document 9] Immunology Today, 1992. 13(12): p. 501 to 507.
 [Non-Patent Document 10] Journal of Allergy and Clinical Immunology, 2013. 132(4): p. 789 to 801.
 [Non-Patent Document 11] Journal of Allergy and Clinical Immunology, 2014. 134(3): p. 671 to 678.

Disclosure of Invention

Problems to Be Solved by the Invention

[0015] A plurality of human CRTH2 antibodies have been established so far, but there has been a demand for establishment of an anti-human CRTH2 antibody having desired activity such as reactivity to various human immune cells, specific binding activity to human CRTH2, or an effect on human CRTH2 ligand-dependent activity.

[0016] An object of the present invention is to provide an anti-human CRTH2 antibody which has desired activity by recognizing and binding to a specific epitope of human CRTH2; the antibody fragment thereof; a DNA which encodes the amino acid sequence of the antibody; a vector which comprises the DNA; hybridomas and antibody producing cells which produce the antibody; a method of producing the antibody; a composition which comprises the antibody or the antibody fragment thereof; a treatment method and a diagnostic method for an allergic disease, an autoimmune disease, a disease accompanied by increase or hyperergasia of eosinophilis, and a disease accompanied by increase or hyperergasia of Th2 cells using the antibody or the antibody fragment thereof; and a medicine and a diagnostic agent which comprise the antibody or the antibody fragment thereof.

Means for Solving the Problems

[0017] The present invention relates to the following (1) to (26).

(1) An antibody or the antibody fragment thereof which recognizes at least one of 192th glycine and 194th aspartic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2 and binds thereto.

(2) The antibody or the antibody fragment thereof according to (1), wherein the antibody recognizes at least one amino acid residue selected from the group consisting of 12th proline, 13th isoleucine, 14th leucine, 15th glutamic acid, 177th aspartic acid, 178th glycine, 179th arginine, 180th isoleucine, 181th methionine, 182nd cysteine, 183rd tyrosine, 184th tyrosine, 185th asparagine, 186th valine, 187th leucine, 188th leucine, 189th leucine, 195th arginine, 196th aspartic acid, 197th alanine, and 198th threonine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2 and binds thereto.

(3) The antibody or the antibody fragment thereof according to (1) or (2), wherein the antibody recognizes at least one amino acid residue selected from the group consisting of the following (a) to (g):

(a) 12th proline, 14th leucine, and 15th glutamic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2;

(b) 177th aspartic acid, 178th glycine, and 179th arginine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2;

(c) 180th isoleucine and 181st methionine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2;

(d) 183rd tyrosine, 184th tyrosine, and 185th asparagine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2;

- (e) 187th leucine, 188th leucine, and 189th leucine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2;
- (f) 195th arginine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2; and
- (g) 196th aspartic acid and 198th threonine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2.

(4) The antibody or the antibody fragment thereof according to any one of (1) to (3), wherein the antibody is any one selected from the group consisting of the following (a) to (d):

- (a) an antibody in which complementarity determining regions (hereinafter, abbreviated as CDR) 1 to 3 of an antibody heavy chain variable region (hereinafter, abbreviated as VH) comprise amino acid sequences represented by SEQ ID NOS: 20 to 22, respectively, and CDRs 1 to 3 of an antibody light chain variable regions (hereinafter, abbreviated as VL) comprise amino acid sequences represented by SEQ ID NOS: 23 to 25, respectively;
- (b) an antibody which comprises VH comprising the amino acid sequence represented by SEQ ID NO: 49 or the amino acid sequence obtained by introducing at least one modification selected from modifications of substituting 18th leucine with methionine, 77th asparagine with serine, 93th valine with threonine, and 117th threonine with valine in the amino acid sequence represented by SEQ ID NO: 49 and VL comprising the amino acid sequence represented by SEQ ID NO: 33 or the amino acid sequence obtained by at least one modification selected from modifications of substituting 2nd isoleucine with valine, 4th methionine with leucine, 15th proline with leucine, and 85th alanine with proline in the amino acid sequence represented by SEQ ID NO: 33;
- (c) an antibody which comprises VH comprising any one of the amino acid sequences represented by SEQ ID NOS: 49, 51, 53, 55, 57, and 59 and VL comprising any one of the amino acid sequences represented by SEQ ID NOS: 33, 35, 37, 39, 41, 43, 45, and 47; and
- (d) an antibody which comprises VH comprising the amino acid sequence represented by SEQ ID NO: 17 and VL comprising the amino acid sequence represented by SEQ ID NO: 19.

(5) The antibody or the antibody fragment thereof according to any one of (1) to (4), wherein the antibody has at least one characteristic selected from the group consisting of the following (a) to (h):

- (a) reactivity of the antibody against human CRTH2 does not decrease in the presence of a ligand of human CRTH2;
- (b) neutralizing activity is not shown by the antibody;
- (c) antibody-dependent cell-mediated cytotoxicity (ADCC) is shown by the antibody;
- (d) a reaction of the antibody with at least one of mast cells and Th1 cells does not occur;
- (e) a reaction of the antibody with at least one selected from eosinophils, basophils, Th2 cells, and type 2 innate lymphoid cells (ILC2) occurs;
- (f) agonist activity is not shown by the antibody;
- (g) a signal caused by the ligand of human CRTH2 is not enhanced by the antibody; and
- (h) reactivity of the antibody against human CRTH2 in an activated or inactivated state does not change.

(6) The antibody or the antibody fragment thereof according to any one of (1) to (5), wherein the antibody comprises a human Fc region.

(7) The antibody or the antibody fragment thereof according to any one of (1) to (6), wherein the antibody is a monoclonal antibody.

(8) The antibody or the antibody fragment thereof according to any one of (1) to (7), wherein the antibody is a recombinant antibody.

(9) The recombinant antibody or the antibody fragment thereof according to (8), wherein the recombinant antibody is any one recombinant antibody selected from a human chimeric antibody, a human CDR-grafted antibody, and a human antibody.

(10) The antibody or the antibody fragment thereof according to any one of (1) to (9), wherein the antibody is an antibody which binds to monkey CRTH2.

(11) The antibody fragment according to any one of (1) to (10), wherein the antibody fragment is any one selected from Fab, Fab', F(ab')₂, scFv, diabody, dsFv, and peptide including CDR.

(12) A hybridoma which generates the antibody or the antibody fragment thereof according to any one of (1) to (11).

(13) ADNA which encodes the antibody or the antibody fragment thereof according to any one of (1) to (11).

(14) A recombinant vector which comprises the DNA according to (13).

(15) A transformant which is obtained by introducing the recombinant vector according to (14) into a host cell.

(16) A method of producing the antibody or the antibody fragment thereof according to any one of (1) to (11), comprising:

culturing the hybridomas according to (12) or the transformant according to (15) in a culture medium;
producing and accumulating the antibody or the antibody fragment thereof according to any one of (1) to (11) in a culture; and
collecting the antibody or the antibody fragment thereof from the culture.

(17) A therapeutic agent for a disease associated with human CRTH2, comprising:

the antibody or the antibody fragment thereof according to any one of (1) to (11) as an active ingredient.

(18) A diagnostic agent for a disease associated with human CRTH2, comprising:

the antibody or the antibody fragment thereof according to any one of (1) to (11) as an active ingredient.

(19) The agent according to (17) or (18), wherein the disease associated with CRTH2 is an allergic disease; an autoimmune disease; a disease accompanied by at least one of increase and hyperergasia of eosinophils; a disease accompanied by at least one of increase and hyperergasia of Th2 cells; or a disease accompanied by at least one of increase and hyperergasia of type 2 innate lymphoid cells (ILC2).

(20) A method of treating a disease associated with human CRTH2 comprising:

administering an effective amount of the antibody or the antibody fragment thereof according to any one of (1) to (11).

(21) A method of diagnosing a disease associated with human CRTH2 comprising:

administering an effective amount of the antibody or the antibody fragment thereof according to any one of (1) to (11).

(22) The method according to (20) or (21), wherein the disease associated with human CRTH2 is an allergic disease; an autoimmune disease; a disease accompanied by at least one of increase and hyperergasia of eosinophils; a disease accompanied by at least one of increase and hyperergasia of Th2 cells; or a disease accompanied by at least one of increase and hyperergasia of ILC2.

(23) The antibody or the antibody fragment thereof according to any one of (1) to (11), for a use in at least one of treatment and diagnosis for a disease associated with human CRTH2.

(24) The antibody or the antibody fragment thereof according to (23), wherein the disease associated with human CRTH2 is an allergic disease; an autoimmune disease; a disease accompanied by at least one of increase and hyperergasia of eosinophils; a disease accompanied by at least one of increase and hyperergasia of Th2 cells; or a disease accompanied by at least one of increase and hyperergasia of ILC2.

(25) Use of the antibody or the antibody fragment thereof according to any one of (1) to (11), for the manufacture of at least one of a therapeutic agent and a diagnostic agent for a disease associated with human CRTH2.

(26) The use according to (25), wherein the disease associated with human CRTH2 is an allergic disease; an autoimmune disease; a disease accompanied by at least one of increase and hyperergasia of eosinophils; a disease accompanied by at least one of increase and hyperergasia of Th2 cells; or a disease accompanied by at least one of increase and hyperergasia of ILC2.

Effects of the Invention

[0018] According to the present invention, an antibody or the antibody fragment thereof or the like which recognizes at least one of 192th glycine and 194th aspartic acid in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2 and binds thereto can be provided.

[0019] The antibody of the present invention specifically reacts with cells expressing CRTH2 such as eosinophils, basophils, Th2 cells, or ILC2, exhibits high reactivity to CRTH2-expressing cells even in the presence of high concentration of a ligand, and does not have agonist activity, neutralizing activity, or activity of enhancing the signal caused by the ligand of human CRTH2. Therefore, the antibody or the antibody fragment thereof of the present invention is capable of exhibiting therapeutic effects that target the cells expressing CRTH2 such as eosinophils, basophils, Th2 cells, or ILC2 which express CRTH2.

Brief Description of the Drawings

[0020]

[Fig. 1] Fig. 1 shows amino acid sequences of the light chain variable region of Lym2 antibody and the light chain variable region of each humanized Lym2 antibody (LV0, LV1, LV2a, LV2b, LV2c, LV3a, LV3b, and LV4) which do not include signal sequences. The regions surrounded by frames in each sequence show CDR sequences.

[Fig. 2] Fig. 2 shows amino acid sequences of the heavy chain variable region of Lym2 antibody and the heavy chain variable region of each humanized Lym2 antibody (HV0, HV1, HV2a, HV2b, HV3, and HV4) which do not

include signal sequences. The regions surrounded by frames in each sequence show CDR sequences.

[Fig. 3] Figs. 3(A) to 3(C) show the results of analyzing cytotoxic activity against human eosinophils and human basophils of rat/human chimeric Lym2 antibody (hereinafter, also referred to as chLym2) and humanized Lym2 antibodies using flow cytometry. In Figs. 3(A) to 3(C), each of the figures on the left side shows cytotoxic activity against human eosinophils and each of the right figures shows cytotoxic activity against human basophils. The vertical axis in each figure represents the number of cells per 2000 control beads and the horizontal axis in each figure represents the antibody concentration. In Fig. 3(A), ○ represents chLym2, ● represents a humanized Lym2 antibody LV0HV0, and Δ represents an isotype control antibody. In Fig. 3(B), ○ represents chLym2, ■ represents a humanized Lym2 antibody LV0HV1, and Δ represents an isotype control antibody. In Fig. 3(C), ○ represents chLym2, ▲ represents a humanized Lym2 antibody LV0HV2a, and Δ represents an isotype control antibody.

[Fig. 4] Fig. 4(A) shows reactivity of a humanized Lym2 antibody LV0HV1 against each of cells which express mutated human CRTH2 with amino acid substitution and Fig. 4(B) shows reactivity of chLym2 against each of cells which express mutated human CRTH2 with amino acid substitution. In each figure, the vertical axis represents the value (%) of reactivity against each of cells which express mutated human CRTH2 with amino acid substitution wherein the value of reactivity, which is relative fluorescent intensity obtained by correcting fluorescent intensity of each anti-human CRTH2 monoclonal antibody with fluorescent intensity of Azami-Green tag, against wild-type human CRTH2-expressing cells is set to 100%. In each horizontal axis, WT represents wild-type human CRTH2 and the others represent mutants with amino acid substitution. The symbol "*" means that 90% or greater of relative fluorescent intensity decreases from the relative fluorescent intensity of wild-type CRTH2. Hereinafter, the same applies to Figs. 5 to 7.

[Fig. 5] Fig. 5(A) shows reactivity of hu19A2 v52 against each of CRTH2 mutants with amino acid substitution and Fig. 5(B) shows reactivity of hu8B1 v1 against each of CRTH2 mutants with amino acid substitution.

[Fig. 6] Fig. 6(A) shows reactivity of ch3C12 against each of CRTH2 mutants with amino acid substitution and Fig. 6(B) shows reactivity of ch31A5 against each of CRTH2 mutants with amino acid substitution.

[Fig. 7] Fig. 7 shows reactivity of BM16 against each of CRTH2 mutants with amino acid substitution.

[Fig. 8] Fig. 8 shows the results of analyzing reactivity of an anti-human CRTH2 monoclonal antibody against human eosinophils using flow cytometry. ● represents Lym2 antibody, ○ represents BM16, ▲ represents 301108. The vertical axis represents fluorescent intensity, and the horizontal axis represents the antibody concentration of each anti-human CRTH2 monoclonal antibody.

[Fig. 9] Fig. 9 shows the results of analyzing reactivity of chLym2 against human basophils using flow cytometry. A portion painted with black represents reactivity of an isotype control antibody and a portion surrounded by a solid line represents reactivity of chLym2. The vertical axis represents the number of cells and the horizontal axis represents the fluorescent intensity.

[Fig. 10] Fig. 10 shows the results of analyzing reactivity of a humanized Lym2 antibody LV0HV1 against human CD4-positive T-cells using flow cytometry. The vertical axis represents fluorescent intensity of fluorescence staining using a humanized Lym2 antibody LV0HV1 against a cell population in which lymphocytes are fractionated by Forward scatter (hereinafter, referred to as FSC)-Side scatter (hereinafter, referred to as SSC) deployment and further fractionated by CD3-positive cells and CD4-positive cells and the horizontal axis represents fluorescent intensity of fluorescence staining resulting from a CD4 antibody with respect to a cell population in which lymphocytes are fractionated by FSC-SSC deployment and further fractionated by CD3-positive cells and CD4-positive cells.

[Fig. 11] Fig. 11(A) and Fig. 11(B) show the results of analyzing cytotoxic activity of an anti-human CRTH2 monoclonal antibody against human eosinophils and human basophils using flow cytometry. The upper figure shows cytotoxic activity against human eosinophils and the lower figure shows cytotoxic activity against human basophils. In each figure, the vertical axis represents the number of cells per 1000 control beads and the horizontal axis represents the antibody concentration, ○ represents a humanized Lym2 antibody LV0HV1, □ represents hu109A2 v52, ▲ represents hu8B1 v1, Δ represents ch3C12, ◆ represents ch31A5, and × represents an isotype control antibody.

[Fig. 12] Fig. 12(A) and Fig. 12(B) show the results of analyzing activity of reducing human Th2 and Th1 cytokine of an anti-human CRTH2 monoclonal antibody. In Fig. 12(A), the vertical axis represents the concentration of IL-5 or IL-13 which is Th2 cytokine under the condition that each antibody is added. Further, in Fig. 12(B), the vertical axis represents the concentration of IFN-γ which is Th1 cytokine under the condition that each antibody is added.

[Fig. 13] Figs. 13(A) to 13(C) show results of analyzing a change in reactivity of an anti-human CRTH2 monoclonal antibody in the presence of DKPGD2 which is a CRTH2 ligand using human CRTH2 expressing 293 EBNA cells using flow cytometry. When the concentrations of the anti-human CRTH2 monoclonal antibody shown in the legends are 0.3 μg/mL, 1 μg/mL, and 3 μg/mL, the results thereof are shown in Fig. 13(A), Fig. 13(B), and Fig. 13(C), respectively. In each figure, the vertical axis represents the percentage of fluorescent intensity when the fluorescent intensity in the absence of DKPGD2 is set to 100%.

[Fig. 14] Fig. 14 shows the results of analyzing reactivity of an anti-human CRTH2 monoclonal antibody against human differentiation induced mast cells stimulated by IgE and a crosslinking antibody treatment using flow cytometry.

etry. Each figure shows reactivity of an antibody described on the top of each figure. The vertical axis represents the number of cells and the horizontal axis represents the fluorescent intensity. A portion painted with black represents reactivity of an isotype control antibody and a portion surrounded by a solid line represents reactivity of the anti-human CRTH2 monoclonal antibody.

[Fig. 15] Fig. 15 shows the results of analyzing reactivity of an anti-human CRTH2 monoclonal antibody against human differentiation induced Th1 cells using flow cytometry. Each figure shows reactivity of an antibody described on the top of each figure. The vertical axis represents the number of cells and the horizontal axis represents the fluorescent intensity. A portion painted with black represents reactivity of an isotype control antibody and a portion surrounded by a solid line represents reactivity of the anti-human CRTH2 monoclonal antibody.

[Fig. 16] Fig. 16 shows the results of evaluation of antagonist activity of Lym2 antibody using shape change of human eosinophils as an index. The vertical axis represents the percentage (%) of eosinophils detected in a high FSC region by flow cytometer analysis when DKPGD2 was treated at the concentrations shown in the legends in the presence or absence of each antibody described on the bottom of the graph.

[Fig. 17] Fig. 17 shows the results of evaluation of antagonist activity of Lym2 antibody using shape change of human eosinophils as an index. The vertical axis represents the percentage (%) of eosinophils detected in a high FSC region when the Lym2 antibody was treated at the concentrations shown in the legends.

[Fig. 18] Figs. 18(A) to 18(C) all show the results of evaluation of agonist activity, antagonist activity and enhancing activity of ligand-induced activation of an anti-human CRTH2 monoclonal antibody using shape change of human eosinophils as an index. Fig. 18(A) shows the result regarding a humanized antibody or a chimeric antibody, Fig. 18(B) shows the result regarding a rat antibody, and Fig. 18(C) shows the result regarding a mouse antibody. In each figure, the vertical axis represents the percentage (%) of eosinophils detected in a high FSC region by flow cytometer analysis when each anti-human CRTH2 monoclonal antibody or isotype antibody shown in the legends is treated in the presence or absence of DKPGD2.

[Fig. 19] Fig. 19 shows the results of analyzing an effect of a change in conformation of CRTH2, caused by GTP γ S or GDP treatment applied to membrane fraction of CRTH2-expressing cells, on the reactivity of a CRTH2 monoclonal antibody by ELISA. The vertical axis represents Fold change where the absorbance when GTP γ and GDP are untreated is set to 1. The horizontal axis represents the presence or absence of GTP γ S and GDP treatment and evaluated antibodies (hu19A2 v52 and a humanized Lym2 antibody LV0HV1).

[Fig. 20] Fig. 20 shows the results of analyzing Azami-Green expression in Azami-Green fusion human CRTH2 expressing CHO/DG44 cells and cynomolgus monkey CRTH2 expressing CHO/DG44 cells using flow cytometry. The vertical axis represents the number of cells and the horizontal axis represents fluorescent intensity of Azami-Green. A portion painted with black represents fluorescent intensity in CHO/DG44 cells which are parent cells, a portion surrounded by a solid line represents fluorescent intensity in Azami-Green fusion human CRTH2 expressing CHO/DG44 cells, and a portion surrounded by a dotted line represents fluorescent intensity in Azami-Green fusion cynomolgus monkey CRTH2 expressing CHO/DG44 cells.

[Fig. 21] Fig. 21 shows the results of analyzing reactivity of a humanized Lym2 antibody LV0HV1 and an isotype antibody against Azami-Green fusion human CRTH2 expressing CHO/DG44 cells and cynomolgus monkey CRTH2 expressing CHO/DG44 cells using flow cytometry. ○ represents reactivity of LV0HV1 against Azami-Green fusion human CRTH2 expressing CHO/DG44 cells, ● represents reactivity of LV0NV1 against Azami-Green fusion cynomolgus monkey CRTH2 expressing CHO/DG44 cells, △ represents reactivity of an isotype antibody against Azami-Green fusion human CRTH2 expressing CHO/DG44 cells, and ▲ represents reactivity of an isotype antibody against Azami-Green fusion cynomolgus monkey CRTH2 expressing CHO/DG44 cells. Further, the vertical axis represents fluorescent intensity and the horizontal axis represents the antibody concentration of each antibody.

Embodiments for Carrying Out the Invention

[0021] As human CRTH2 in the present invention, a polypeptide comprising the amino acid sequence represented by SEQ ID NO: 2 or GenBank accession number BAA74518 may be exemplified. A polypeptide comprising an amino acid sequence, in which one or more amino acids are deleted, substituted, inserted, and/or added in the amino acid sequence represented by SEQ ID NO: 2 or GenBank accession number BAA74518, and has a function of human CRTH2 and a polypeptide which is formed of an amino acid sequence having 60% or greater, preferably 80% or greater, more preferably 90% or greater, still more preferably 95% or greater, and most preferably 98% or greater homology with the amino acid sequence represented by SEQ ID NO: 2 or GenBank accession number BAA74518 and having a function of human CRTH2 are incorporated in human CRTH2 of the present invention.

[0022] A polypeptide comprising an amino acid sequence represented by SEQ ID NO: 2 or GenBank accession number BAA74518, in which one or more amino acids are deleted, substituted, inserted, and/or added can be obtained by site-directed mutagenesis [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989), Current Protocols in Molecular Biology, John Wiley & Sons (1987 to 1997), Nucleic Acids Research, 10, 6487

(1982), Proc. Natl. Acad. Sci. USA, 79, 6409 (1982), Gene, 34, 315 (1985), Nucleic Acids Research, 13, 4431 (1985), Proc. Natl. Acad. Sci. USA, 82, 488 (1985)], for example, by introducing site-directed mutation to DNA that encodes a polypeptide comprising the amino acid sequence represented by SEQ ID NO: 2. The number of amino acids to be deleted, substituted, or added is not particularly limited, but preferably in a range of 1 to several tens of amino acids, for example, 1 to 20 amino acids and more preferably in a range of 1 to several amino acids, for example, 1 to 5 amino acids.

[0023] As a gene encoding human CRTH2, a base sequence represented by GenBank accession number AB008535 or SEQ ID NO: 1 may be exemplified. A gene which comprises a base sequence represented by GenBank accession number AB008535 or SEQ ID NO: 1 in which one or more base is deleted, substituted, or added and includes DNA which encodes a protein having a function of human CRTH2; a gene which comprises a base sequence having at least 60% or greater, preferably 80% or greater, and more preferably 95% or greater homology with the base sequence represented by GenBank accession number AB008535 or SEQ ID NO: 1 and comprises DNA which encodes a polypeptide having a function of human CRTH2; and a gene which comprises DNA hybridizing with DNA comprising the base sequence represented by SEQ ID NO: 1 under stringent conditions and comprises DNA encoding a polypeptide having a function of human CRTH2 are included in the genes encoding CRTH2 of the present invention.

[0024] The DNA hybridizing under stringent conditions indicates DNA which is obtained by a colony hybridization method, a plaque hybridization method, a southern blot hybridization method, or a DNA microarray method using DNA comprising the base sequence represented by SEQ ID NO: 1 as a probe and is capable of hybridizing.

[0025] Specifically, DNA which can be identified by performing hybridization at 65°C in the presence of 0.7 to 1.0 mol/L of sodium chloride using a filter or a slide glass with a hybridized colony- or plaque-derived DNA, a PCR product comprising the sequence or an oligo DNA immobilizing there on, and then washing the filter or slide glass at 65°C with 0.1 to 2-fold concentration of SSC solution (SSC solution having a 1-fold concentration consists of 150 mmol/L of sodium chloride and 15 mmol/L of sodium citrate) [Molecular cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989), Current Protocols in Molecular Biology, John Wiley & Sons (1987 to 1997), DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University, (1995)] may be exemplified.

[0026] As DNA which is capable of hybridizing, DNA having at least 60% or greater, preferably 80% or greater, and more preferably 95% or greater homology with the base sequence represented by GenBank accession number AB008535 or SEQ ID NO: 1 may be exemplified.

[0027] In a base sequence of a gene which encodes protein of a eukaryote, gene polymorphism is frequently found. A gene in which a base sequence of genes used in the present invention is mutated in a small scale by such polymorphism is also included in genes encoding human CRTH2 of the present invention.

[0028] The value of homology in the present invention may be a value calculated using a homology search program known to those skilled in the art unless otherwise noted. Further, in regard to base sequences, values calculated using default parameters in BLAST [J. Mol. Biol., 215, 403 (1990)] and the like may be exemplified. In regard to amino acid sequences, values calculated using default parameters in BLAST2 [Nucleic Acids Res., 25, 3389 (1997), Genome Res., 7, 649 (1997), <http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/information3.html>] and the like may be exemplified.

[0029] As the default parameters, G (Cost to open gap) is 5 for a base sequence and 11 for an amino acid sequence; -E (Cost to extend gap) is 2 for a base sequence and 1 for an amino acid sequence; -q (Penalty for nucleotide mismatch) is -3; -r (reward for nucleotide match) is 1; -e (expect value) is 10, -W (wordsize) is 11 residues for a base sequence and 3 residues for an amino acid sequence; -y [Dropoff (X) for blast extensions in bits] is 20 for blastn and 7 for a program other than blastn; -X (X dropoff value for gapped alignment in bits) is 15; and -Z (final X dropoff value for gapped alignment in bits) is 50 for blastn and 25 for a program other than blastn (<http://www.ncbi.nlm.nih.gov/blast/html/blastcgihelp.html>).

[0030] The polypeptide comprising a partial sequence of the amino acid sequence represented by SEQ ID NO: 2 or GenBank accession number BAA74518 can be produced by a method known to those skilled in the art. For example, it can be produced by deleting a part of DNA encoding the amino acid sequence represented by SEQ ID NO: 2 and culturing a transformant into which an expression vector containing the DNA is introduced. In addition, using the same method as described above, a polypeptide comprising an amino acid sequence represented by the partial sequence of the amino acid represented by SEQ ID NO: 2 or GenBank accession number BAA74518 in which one or more amino acids are deleted, substituted, or added can be obtained based on the polypeptide or the DNA produced according to the above-described method. Moreover, the polypeptide comprising a partial sequence of the amino acid sequence represented by SEQ ID NO: 2 or GenBank accession number BAA74518 or the polypeptide comprising the partial sequence of the amino acid represented by SEQ ID NO: 2 or GenBank accession number BAA74518 in which one or more amino acids are deleted, substituted, or added can be produced using a chemical synthesis method such as a fluorenylmethyloxycarbonyl (Fmoc) method or a t-butyloxycarbonyl (tBoc) method.

[0031] Examples of the functions of human CRTH2 include migration of cells expressing human CRTH2, enhancement of production of cytokine from the cells, and induction of a change in cell shape accompanied by a change in diameter or surface area in cells by human CRTH2-dependent intracellular signal transduction caused by binding to the ligand thereof, for example, PGD2.

[0032] Examples of extracellular regions of human CRTH2 include an N-terminal region comprising 1 st to 33th amino

acid residues; a loop 1 region comprising 95th to 111st amino acid residues; a loop 2 region comprising 169th to 206th amino acid residues; and a loop 3 region comprising 264th to 285th amino acid residues [J Immunol, 1999. 162(3): p. 1278 to 1286] in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2. As the N-terminal region, the loop 1 region, the loop 2 region, and the loop 3 region, specifically, polypeptide portions comprising 1st to 33th amino acid residues, 95th to 111th amino acid residues, 169th to 206th amino acid residues, and 264th to 285th amino acid residues in the amino acid sequence represented by SEQ ID NO: 2, respectively, may be exemplified.

[0033] The antibody of the present invention may be any kind of antibody such as a monoclonal antibody or a polyclonal antibody, but a monoclonal antibody is preferable. Specific examples of the antibody of the present invention include an antibody produced by hybridomas or a recombinant antibody produced by a recombinant DNA technology. Further, examples of the recombinant antibody include a mouse antibody, a rat antibody, a human chimeric antibody, a humanized antibody, and a human antibody produced by a recombinant DNA technology.

[0034] The monoclonal antibody is an antibody secreted by antibody-producing cells of a single clone and recognizes only one epitope (also referred to as antigenic determinant), and amino acid sequences (primary structure) constituting the monoclonal antibody are uniform.

[0035] Examples of the monoclonal antibody of the present invention include recombinant antibodies produced using a recombinant DNA technology such as an antibody produced by hybridomas and an antibody produced by a transformant transformed by an expression vector including antibody genes.

[0036] The polyclonal antibody is an antibody group including two or more monoclonal antibodies and a plurality of epitopes can be recognized by a plurality of antibodies constituting the antibody group.

[0037] Example of the epitope of the present invention include a single amino acid sequence, a three-dimensional structure comprising the amino acid sequence, an amino acid sequence modified by post-translational modification, and a three-dimensional structure comprising the amino acid sequence, and the like which is recognized and bound by the antibody.

[0038] As the amino acid sequence modified by post-translational modification, an amino acid sequence which is bound by an O-binding type sugar chain in which sugar chain binds to threonine and serine which have a OH substituent, a N-binding type sugar chain in which a sugar chain binds to glutamine and asparagine which have a NH₂ substituent, a sulfate group in which a sulfuric acid molecule binds to threonine which has a OH substituent, or the like may be exemplified.

[0039] The epitope of human CRTH2 recognized by the antibody of the present invention can be determined by performing a binding experiment of an antibody using a deletion variant obtained by deleting a domain as a part of human CRTH2, a mutant obtained by substituting an amino acid residue as a part of human CRTH2 with another amino acid residue, a mutant substituted with a domain derived from other proteins, and a partial peptide fragment of human CRTH2 and the like. Further, the epitope of human CRTH2 to which the antibody of the present invention binds can be determined by adding the antibody of the present invention to human CRTH2 digested by a protease and performing epitope mapping using known mass spectrometry.

[0040] As the amino acid residue included in the epitope of human CRTH2 recognized by the antibody of the present invention, an amino acid residue identified by the loss of reactivity of the antibody of the present invention due to substitution of the amino acid residue may be exemplified.

[0041] The reactivity of the antibody of the present invention can be determined, for example, by measuring the amount (corrected according to the amount of the wild-type human CRTH2 receptor or the mutant with amino acid substitution to be expressed) of the antibody which bind to cells expressing a wild-type human CRTH2 receptor or a mutant with amino acid substitution using flow cytometry or the like. Further, the amount of the antibody which bind can be confirmed by radioimmunoassay using a solid-phase sandwich method or the like; a known immunological detection method with respect to human CRTH2 using an enzyme immunoassay (ELISA) method or the like; or a surface plasmon resonance method using a Biacore system (GE Healthcare) or the like.

[0042] Further, the amount can be confirmed by combining a known immunological detection method [Monoclonal Antibodies-Principles and Practice, Third edition, Academic Press (1996), Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988), monoclonal antibody experiment manual, Kodansha Scientific (1987)] or the like.

[0043] The expression of "the reactivity of the antibody of the present invention is lost" means that the reactivity of the antibody against cells expressing a mutant with amino acid substitution decreases by 70% or greater, preferably 80% or greater, more preferably 90% or greater, and still more preferably 95% or greater, compared to reactivity of the antibody against cells expressing wild-type human CRTH2.

[0044] As the epitope to which the antibody of the present invention binds, an epitope comprising at least one amino acid residue of 192th glycine and 194th aspartic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO:2 may be exemplified.

[0045] Moreover, specific examples of the epitope to which the antibody of the present invention binds include the following epitopes (a) to (c).

- (a) an epitope comprising 192th glycine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2;
 (b) an epitope comprising 194th aspartic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2; and
 (c) an epitope comprising 192th glycine and 194th aspartic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2.

[0046] Moreover, examples of the epitope to which the antibody of the present invention binds include an epitope which comprises at least one amino acid residue of 192nd glycine and 194th aspartic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2 and comprises at least one amino acid residue selected from the group consisting of 12th proline, 14th leucine, 15th glutamic acid, 177th aspartic acid, 178th glycine, 179th arginine, 180th isoleucine, 181st methionine, 183rd tyrosine, 184th tyrosine, 185th asparagine, 187th leucine, 188th leucine, 189th leucine, 195th arginine, 196th aspartic acid, and 198th threonine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2.

[0047] Moreover, examples of the epitope to which the antibody of the present invention binds include an epitope which comprises at least one amino acid residue of 192th glycine and 194th aspartic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2 and includes at least one of the following (a) to (g).

(a) 12th proline, 14th leucine, and 15th glutamic acid in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2;

(b) 177th aspartic acid, 178th glycine, and 179th arginine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2;

(c) 180th isoleucine and 181st methionine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2;

(d) 183rd tyrosine, 184th tyrosine, and 185th asparagine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2;

(e) 187th leucine, 188th leucine, and 189th leucine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2;

(f) 195th arginine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2; and

(g) 196th aspartic acid and 198th threonine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2.

[0048] Other amino acid residue included in the epitope to which the antibody of the present invention binds is not particularly limited as long as the amino acid residue is present in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2 and substantially recognized and binded when the antibody of the present invention binds to human CRTH2, and specific examples thereof include an amino acid residue present, on the three-dimensional structure, in the vicinity of the amino acid residue selected from the group consisting of 12th proline, 14th leucine, 15th glutamic acid, 177th aspartic acid, 178th glycine, 179th arginine, 180th isoleucine, 181st methionine, 183rd tyrosine, 184th tyrosine, 185th asparagine, 187th leucine, 188th leucine, 189th leucine, 192nd glycine, 194th aspartic acid, 195th arginine, 196th aspartic acid, and 198th threonine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2; and an amino acid residue present, in the primary sequence, in the vicinity of the amino acid sequence selected from amino acids selected from the group consisting of 12th proline, 14th leucine, 15th glutamic acid, 177th aspartic acid, 178th glycine, 179th arginine, 180th isoleucine, 181st methionine, 183rd tyrosine, 184th tyrosine, 185th asparagine, 187th leucine, 188th leucine, 189th leucine, 192nd glycine, 194th aspartic acid, 195th arginine, 196th aspartic acid, and 198th threonine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2.

[0049] An antibody molecule is also referred to as immunoglobulin (hereinafter, referred to as Ig) and human antibodies are classified into isotypes of IgA1, IgA2, IgD, IgE, IgG1, IgG2, IgG3, IgG4, and IgM according to a difference in molecular structure. IgG1, IgG2, IgG3, and IgG4 having relatively high homology of an amino acid sequence are also collectively referred to as IgG.

[0050] An antibody molecule is constituted with a polypeptide referred to as a heavy chain (hereinafter, referred to as an H chain) and a polypeptide referred to as a light chain (hereinafter, referred to as an L chain). Further, the H chain is constituted with each region of an H chain variable region (also referred to as VH) and an H chain constant region (also referred to as CH) from the N terminal side and the L chain is constituted with each region of an L chain variable region (also referred to as VL) and an L chain constant region (also referred to as CL) from the N terminal side.

[0051] In the CH, an α chain, a δ chain, a ϵ chain, a γ chain, and a μ chain are known for each sub-class. The CH is constituted with each domain of a CH1 domain, a hinge domain, a CH2 domain, and a CH3 domain from the N terminal side. A domain indicates a functional structural unit constituting each polypeptide of an antibody molecule. Moreover, the CH2 domain and the CH3 domain are collectively referred to as an Fc region or simply referred to as Fc. In the CL, a C λ chain and a C κ chain are known.

[0052] The CH in the antibody of the present invention is not particularly limited as long as the CH belongs to Ig, but any one of the IgG class is preferable and any one in the sub-class such as IgG1, IgG2, IgG3, or IgG4 belonging to the IgG class can be also used.

[0053] The amino acid sequence of the CL in the antibody of the present invention may be any of an amino acid sequence of a human antibody or an amino acid sequence of a non-human animal antibody, but C κ or C λ of an amino acid sequence of a human antibody is preferable.

[0054] The antibody of the present invention is an antibody that recognizes at least one amino acid residue of 192th glycine and 194th aspartic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2 and binds thereto.

[0055] Specific examples of the antibody of the present invention include an antibody selected from the following (a) to (c).

(a) An antibody which recognizes 192th glycine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2 and binds thereto;

(b) An antibody which recognizes 194th aspartic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2 and binds thereto; and

(c) An antibody which recognizes both of 192th glycine and 194th aspartic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2 and binds thereto.

[0056] Further, as the antibody of the present invention, an antibody which recognizes at least one amino acid residue of 192nd glycine and 194th aspartic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2 and recognizes at least one amino acid residue selected from the group consisting of 12nd proline, 14th leucine, 15th glutamic acid, 177th aspartic acid, 178th glycine, 179th arginine, 180th isoleucine, 181st methionine, 183rd tyrosine, 184th tyrosine, 185th asparagine, 187th leucine, 188th leucine, 189th leucine, 195th arginine, 196th aspartic acid, and 198th threonine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2 and binds thereto may be exemplified.

[0057] Further, as the antibody of the present invention, an antibody which recognizes at least one amino acid residue of 192th glycine and 194th aspartic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2 and recognizes at least one of the following (a) to (g) and binds thereto may be exemplified.

(a) 12th proline, 14th leucine, and 15th glutamic acid in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2;

(b) 177th aspartic acid, 178th glycine, and 179th arginine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2;

(c) 180th isoleucine and 181th methionine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2;

(d): 183th tyrosine, 184th tyrosine, and 185th asparagine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2;

(e) 187th leucine, 188th leucine, and 189th leucine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2;

(f) 195th arginine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2; and

(g) 196th aspartic acid and 198th threonine in the amino acid sequences of human CRTH2 represented by SEQ ID NO: 2.

[0058] Specific examples of the antibody of the present invention include an antibody selected from the following (a) to (d).

(a) An antibody in which the amino acid sequences of complementary determining regions (CDRs, hereinafter, abbreviated as CDRs) 1 to 3 of VH comprises the amino acid sequences represented by SEQ ID NOS: 20, 21, and 22, respectively, and the amino acid sequences of CDRs 1 to 3 of VL includes the amino acid sequences represented by SEQ ID NOS: 23, 24, and 25, respectively;

(b) An antibody which competes with the above-described antibody (a) and binds to human CRTH2;

(c) An antibody which binds to an epitope having an epitope to which the above-described antibody (a) binds; and

(d) An antibody which binds to the same epitope as the epitope to which the above-described antibody (a) binds.

[0059] The above-described antibody (b) of the present invention indicates an anti-human CRTH2 antibody which inhibits the binding of the above-described antibody (a) to human CRTH2. Further, in a case where the above-described antibody (a) is set to a first antibody and the epitope to which the first antibody binds is set to a first epitope, the above-

described antibody (c) of the present invention indicates an antibody which binds to an epitope having the first epitope.

[0060] Further, specific examples of the antibody of the present invention include an antibody selected from the following (a) to (c).

(a) An antibody which comprises VH comprising the amino acid sequence represented by SEQ ID NO: 49 or the amino acid sequence which is obtained by introducing at least one modification selected from modifications of substituting 18th leucine with methionine, 77th asparagine with serine, 93th valine with threonine, and 117th threonine with valine in the amino acid sequence represented by SEQ ID NO: 49 and VL comprising the amino acid sequence represented by SEQ ID NO: 33 or the amino acid sequence which is obtained by introducing at least one modification selected from modifications of substituting 2th isoleucine with valine, 4th methionine with leucine, 15th proline with leucine, and 85th alanine with proline in the amino acid sequence represented by SEQ ID NO: 33;

(b) An antibody which comprises VH comprising any one of the amino acid sequences represented by SEQ ID NOS: 49, 51, 53, 55, 57, and 59 and VL comprising any one of the amino acid sequences represented by SEQ ID NOS: 33, 35, 37, 39, 41, 43, 45, and 47; and

(c) An antibody which comprises VH comprising the amino acid sequence represented by SEQ ID NO: 17 and VL comprising the amino acid sequence represented by SEQ ID NO: 19.

[0061] Preferred examples of the above-described antibody (b) include an antibody selected from the following (1) to (3).

(1) An antibody which comprises VH comprising the amino acid sequence represented by SEQ ID NO: 49 and VL comprising any one of the amino acid sequences represented by SEQ ID NOS: 33, 35, 37, 39, 41, 43, 45, and 47;

(2) An antibody which comprises VH comprising the amino acid sequence represented by SEQ ID NO: 59 and VL comprising any one of the amino acid sequences represented by SEQ ID NOS: 33, 35, 37, 39, 41, 43, 45, and 47; and

(3) An antibody which comprises VH comprising any one of the amino acid sequences represented by SEQ ID NOS: 51, 53, 55, and 57 and VL comprising the amino acid sequence represented by SEQ ID NO: 33.

[0062] Particularly preferred examples of the above-described antibody (b) include an antibody which comprises VH comprising the amino acid sequence represented by SEQ ID NO: 51 and VL comprising the amino acid sequence represented by SEQ ID NO: 33.

[0063] As the antibody of the present invention, an antibody whose reactivity against a mutant with amino acid substitution obtained by substituting at least one of 192th glycine and 194th aspartic acid of human CRTH2 with alanine is lost may be exemplified.

[0064] Further, the antibody of the present invention includes an antibody whose reactivity against human CRTH2 does not decrease in the presence of a ligand of human CRTH2. The antibody whose reactivity against human CRTH2 does not decrease in the presence of the ligand of human CRTH2 is capable of exhibiting high reactivity under conditions in which the ligand of human CRTH2 is present at a high concentration such as a local inflammation area, compared to an antibody whose reactivity against human CRTH2 decreases. Accordingly, the antibody can specifically bind to human CRTH2 in a human CRTH2 ligand-independent manner and thus drug efficacy can be exhibited.

[0065] In the present invention, the expression of "the reactivity of the antibody decreases in the presence of a ligand of human CRTH2" means that the reactivity of the antibody against human CRTH2-expressing cells decreases by 5% or greater in the presence of the ligand of human CRTH2 compared to the reactivity in the absence of the ligand of human CRTH2. More precisely, the expression means that the reactivity decreases by 10% or greater.

[0066] The ligand of human CRTH2 is not particularly limited as long as the ligand specifically binds to human CRTH2, and preferred examples thereof include PGD2 and DKPGD2. Among the examples, DKPGD2 is more preferable.

[0067] In the present invention, the expression of "the reactivity against human CRTH2 in the activated or inactivated state does not change" means that the reactivity of the antibody against human CRTH2 does not change in the presence or absence of guanosine diphosphate (GDP), GDP analog, guanosine triphosphate (GTP), or GTP analog.

[0068] As the GDP analog, guanosine 5'-O-(β -thio) diphosphate (GDPPS) may be exemplified. As the GTP analog, guanosine 5'-O-(γ -thio) triphosphate (GTP γ S) may be exemplified.

[0069] The antibody of the present invention include an antibody which does not have neutralizing activity, an antibody which does not have agonist activity, an antibody which does not enhance the signal caused by the ligand of human CRTH2, and an antibody whose reactivity against human CRTH2 in the activated or inactivated state does not change.

[0070] In the present invention, the neutralizing activity of the antibody indicates activity of inhibiting biological activity of human CRTH2 of the antibody. Examples thereof include antagonist activity such as activity of inhibiting binding of human CRTH2 to the ligand thereof or activity of inhibiting transduction of the signal caused by the human CRTH2.

[0071] In the present invention, the agonist activity indicates activity that mimics biological activity of the ligand of human CRTH2 such as activity of inducing activation of CRTH2 or various reactions accompanied by the activation. Specific examples of the agonist activity of the present invention include cell migration activity and activity of inducing

cell shape change.

[0072] In the present invention, the signal caused by the ligand of human CRTH2 indicates the signal as a consequence of activation of human CRTH2 by binding of the ligand of human CRTH2.

[0073] The signal caused by the ligand of human CRTH2 and the agonist activity can be evaluated by analyzing various reactions as a consequence of activation of human CRTH2. For example, the signal and the agonist activity can be evaluated by analyzing shape change of human CRTH2-expressing cells.

[0074] Any cells may be used as human CRTH2-expressing cells as long as the cells express human CRTH2, and examples thereof include eosinophils, basophils, Th2 cells, type 2 innate lymphoid cells (ILC2), non-classical monocyte, and Th2/Th17 cells.

[0075] In the present invention, the expression of "the antibody does not enhance the signal caused by the ligand of human CRTH2" indicates that activation of human CRTH2 and various reactions as a consequence of the activation are not enhanced when human CRTH2 is treated with both of the ligand of human CRTH2, compared to a case where human CRTH2 is treated with only the ligand of human CRTH2.

[0076] The antibody of the present invention includes an antibody exhibiting cytotoxic activity against human CRTH2-expressing cells. Examples of the cytotoxic activity of the present invention include complement-dependent cytotoxic activity (hereinafter, referred to as CDC activity) and antibody-dependent cellular cytotoxic activity (hereinafter, referred to as ADCC activity).

[0077] As the CDC activity of the present invention, a reaction in which an antibody molecule which binds to human CRTH2 on the cell surface binds to C1q of a complement system through an Fc portion and, as a result, each complement component from C1 to C9 is activated so that C5 to C9 finally form a hole-forming polymer referred to as a membrane attack complex on the cell membrane to cause cytolysis may be exemplified [Immunol Today. 1999 Dec; 20(12): 576 to 82]

[0078] As the ADCC activity of the present invention, a cytotoxic response caused by release of cytotoxic molecules such as perforin and granzyme or enhancement of phagocytosis caused by activation of Fc receptor-expressing cells such as natural killer cells (hereinafter, referred to as NK cells) through the Fc portion by binding of an antibody molecule, which binds to human CRTH2 on the cell surface may be exemplified [Chemical Immunology, 65, 88 (1997); Immunol Today, 20, 576 (1999)].

[0079] The antibody of the present invention includes an antibody which does not have cellular cytotoxicity against mast cells. Such an antibody has an advantage that there is no fear of side effect caused by release of an inflammatory mediator due to the injury of the mast cells.

[0080] The antibody of the present invention include an antibody in which a N-glycoside-linked sugar chain binds to an Fc region of the antibody and fucose does not bind to N-acetyl glucosamine of a reducing terminal of the N-glycoside-linked sugar chain. As the antibody in which a N-glycoside-linked sugar chain binds to an Fc region of the antibody and fucose does not bind to N-acetyl glucosamine of a reducing terminal of the N-glycoside-linked sugar chain, an antibody produced using CHO cells (Pamphlet of International Publication No. 2005/035586, Pamphlet of International Publication No. 02/31140) in which α 1,6-fucose transferase genes are deficient may be exemplified. The antibody of the present invention in which a N-glycoside-linked sugar chain binds to an Fc region of the antibody and fucose does not bind to N-acetyl glucosamine of a reducing terminal of the N-glycoside-linked sugar chain has high ADCC activity.

[0081] The antibody of the present invention includes an antibody in which amino acid residues of an Fc region of the antibody are modified such that the binding activity of the amino acid residues to an Fc receptor becomes high. As the antibody in which amino acid residues of an Fc region of the antibody are modified such that the binding activity to an Fc receptor becomes high, and antibody molecules produced by a method described in US Patent No. 7317091 may be exemplified.

[0082] The antibody of the present invention include an antibody in which the surface charge of a polypeptide comprising a variable region of the antibody or an antigen-binding activity in the pH in an early endosome is modified so that half-life in blood is extended.

[0083] As the antibody in which the surface charge of a polypeptide comprising a variable region of the antibody molecule or antigen-binding activity in the pH in an early endosome is modified so that half-life in blood is extended, antibodies produced by the methods described in JP-A-2013-165716 and JP-A-2012-021004A may be exemplified.

[0084] The antibody of the present invention include recombinant antibodies such as a human chimeric antibody (hereinafter, also simply referred to as a chimeric antibody), a human CDR-grafted antibody (hereinafter, also referred to as a humanized antibody), and a human antibody.

[0085] The chimeric antibody indicates an antibody comprising VH and VL of an antibody of animal other than humans (non-human animal) and a CH and a CL of a human antibody. Any animal such as a mouse, a rat, a hamster, or a rabbit can be used as the non-human animal as long as hybridomas can be prepared.

[0086] The chimeric antibody of the present invention can be produced by obtaining cDNA that encodes the VH and the VL of an antibody of a non-human animal specifically reacting with human CRTH2, inserting the cDNA into an expression vector for animal cell comprising genes encoding the CH and the CL of a human antibody, respectively, and thus constructing a chimeric antibody expression vector and by introducing the vector into animal cells to express the

antibody.

[0087] The humanized antibody indicates an antibody in which the CDRs of the VH and the VL of an antibody of an animal other than humans is grafted into suitable positions in the VH and the VL of a human antibody.

[0088] The humanized antibody of the present invention can be produced by constructing cDNA that encodes a variable region (hereinafter, also referred to as a V region) constructed by grafting the CDRs of the VH and the VL of an antibody of a non-human animal specifically reacting with human CRTH2 to a framework (hereinafter, also referred to as an FR) of the VH and the VL of a suitable human antibody, inserting the cDNA into an expression vector for animal cell comprising DNA encoding the CH and the CL, respectively, and thus constructing a humanized antibody expression vector and by introducing the vector into animal cells to express the antibody.

[0089] Any amino acid sequence can be used as the amino acid sequence of the FR of the VH and the VL of a human antibody as long as the amino acid sequence is derived from a human antibody. Specific examples thereof include amino acid sequences of the FR of the VH and the VL of a human antibody registered in the database such as Protein Data Bank and common amino acid sequences of each sub group of the FR of the VH and the VL of a human antibody (Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services, 1991) may be exemplified.

[0090] The antibody of the present invention include antibodies comprising these amino acid sequences in which one or more amino acids are deleted, added, substituted, or inserted and which specifically binds to human CRTH2 and has the same function in the biological activity such as cytotoxic activity; and the antibody fragments.

[0091] The antibody of the present invention includes an antibody bonded to monkey CRTH2. Examples of the monkey CRTH2 include marmoset CRTH2, cynomolgus monkey CRTH2, and rhesus monkey CRTH2. Among these, cynomolgus monkey CRTH2 is preferable.

[0092] The antibody of the present invention include Fc fusion protein in which Fc and an antibody fragment are bound to each other, Fc fusion protein in which Fc and a naturally-occurring ligand or a receptor are bound to each other (also referred to as immunoadhesion), and Fc fusion protein in which a plurality of Fc regions are fused. Further, modified Fc regions including amino acid residue modification in which amino acid residue substitution is carried out for stabilizing an antibody or for controlling half-life in blood can be used for the antibodies of the present invention.

[0093] In the present invention, the antibody fragment is a fragment which comprises an antigen-binding domain which recognizes at least one of 192th glycine and 194th aspartic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2, and binds thereto, and has antigen-binding activity. Examples of the antibody fragment of the present invention include Fab, Fab', F(ab')₂, single chain Fv (hereinafter, also referred to as scFv), diabody, dsFv, and peptides comprising a plurality of CDRs.

[0094] Fab is an antibody fragment with a molecular weight of approximately 50000 in which the approximately half of an H chain on an N terminal side and the entire L chain are bonded to each other with a disulfide bond and which has antigen-binding activity, among fragments obtained by treating IgG with a protease papain (cleaving at the 224th amino acid residue of the H chain).

[0095] The Fab of the present invention can be obtained by treating the antibody of the present invention which specifically binds to human CRTH2 with a protease papain. Alternatively, the Fab can be produced by inserting DNA-encoding Fab of the antibody into an expression vector for prokaryote or an expression vector for eukaryote and introducing the vector into a prokaryote or a eukaryote.

[0096] F(ab')₂ is an antibody fragment with a molecular weight of approximately 100000 which is slightly larger than Fab fragments bonded to each other through a disulfide bond of the hinge region and has antigen-binding activity, among fragments obtained by treating IgG with a protease pepsin (cleaving at the 234th amino acid residue of the H chain is cut).

[0097] The F(ab')₂ of the present invention can be obtained by treating the antibody of the present invention which specifically binds to human CRTH2 with a protease pepsin. Alternatively, the F(ab')₂ can be produced by bonding the following Fab' through a thioether bond or a disulfide bond.

[0098] Fab' is an antibody fragment which is obtained by cutting the disulfide bond of the hinge region of the F(ab')₂ and has antigen-binding activity with a molecular weight of approximately 50000.

[0099] The Fab' of the present invention can be obtained by treating the F(ab')₂ composition which specifically binds to human CRTH2 of the present invention with a reductant dithiothreitol. Alternatively, the Fab' can be produced by inserting DNA encoding Fab' fragment of the antibody into an expression vector for prokaryote or an expression vector for eukaryote and introducing the vector into a prokaryote or a eukaryote to express the Fab'.

[0100] scFv is an antibody fragment having antigen-binding activity, which is a VH-P-VL or VL-P-VH polypeptide in which one VH and one VL are linked to each other using an appropriate peptide linker (P) such as a linker peptide obtained by connecting an arbitrary number of linkers formed of four Glys and one Ser residue (G4S).

[0101] scFv of the present invention can be produced by obtaining cDNA that encodes the VH and the VL of the antibody of the present invention which specifically binds to human CRTH2, constructing DNA which encodes scFv, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote and introducing the vector into a prokaryote or a eukaryote to express the scFv.

[0102] A diabody is an antibody fragment in which scFv is dimerized and which has divalent antigen-binding activity.

The divalent antigen-binding activity may be the same as each other or may be made to be different from each other.

[0103] The diabody of the present invention can be produced by obtaining cDNA that encodes the VH and the VL of an antibody of the present invention which specifically binds to human CRTH2, forming DNA encoding scFv such that the length of the amino acid sequence of P becomes 8 residues or less, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote and introducing the vector into a prokaryote or a eukaryote.

[0104] dsFv is obtained by binding polypeptides in which one amino acid residue in each VH and VL is substituted with a cysteine residue through a disulfide bond between the cysteine residues. The amino acid residue to be substituted with a cysteine residue can be selected based on the three-dimensional structure prediction of an antibody according to a method shown by Reiter et al. (Protein Engineering, 7, 697, 704, 1994).

[0105] The dsFv of the present invention can be produced by obtaining cDNA that encodes the VH and the VL of the antibody of the present invention which specifically binds to human CRTH2, constructing DNA encoding dsFv, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote and introducing the expression vector into a prokaryote or a eukaryote to express the dsFv.

[0106] The peptide comprising CDR is formed to include at least one region of the CDR of VH or VL. The peptide including a plurality of CDRs can be bound to each other directly or through an appropriate peptide linker.

[0107] The peptide comprising CDR of the present invention can be produced by constructing DNA encoding CDR of VH and VL of the antibody of the present invention which specifically bound to human CRTH2, inserting the DNA into an expression vector for prokaryote or a expression vector for eukaryote, and introducing the expression vector into a prokaryote or a eukaryote to express the peptide comprising CDR.

[0108] Moreover, a peptide comprising CDR can be produced by a chemical synthesis method such as a Fmoc method (fluorenylmethyloxycarbonyl method) or a tBoc method (t-butyloxycarbonyl method).

[0109] The antibody or the antibody fragment thereof of the present invention includes a monoclonal antibody or the antibody fragment thereof in which one or more amino acids comprising an amino acid sequence constituting the above-described antibody or the antibody fragment thereof are deleted, substituted, inserted, or added and has the same activity as that of the above-described antibody or the antibody fragment thereof. The number of amino acids to be deleted, substituted, inserted, or added is not particularly limited as long as the number is 1 or greater, but is almost the number of amino acids which can be deleted, substituted, or added by a known technique such as site-specific mutagenesis described in Molecular Cloning, Second Edition, Current Protocols in Molecular Biology, Nucleic Acids Research, 10, 6487 (1982), Proc. Natl. Acad. Sci., USA, 79, 6409 (1982), Gene, 34, 315 (1985), Nucleic Acids Research, 13, 4431 (1985), and Proc. Natl. Acad. Sci. USA, 82, 488 (1985). The number of amino acids may be 1 to several tens, preferably 1 to 20, more preferably 1 to 10, and still more preferably 1 to 5.

[0110] The expression of "one or more amino acid residues are deleted, substituted, inserted, or deleted in the amino acid sequence of human CRTH2 or the antibody of the present invention" means that one or a plurality of amino acid residues are deleted, substituted, inserted or added at optional positions in one or a plurality of amino acid sequences in the same sequence. The deletion, substitution, insertion, or addition may occur simultaneously and amino acid residues to be substituted, inserted, or added may be natural type or non-natural type residues.

[0111] Examples of natural type amino acid residue include L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, and L-cysteine.

[0112] Preferred examples of the amino acid residues which can be substituted with each other are as follows. Amino acid residues included in the same group can be substituted with each other.

A group: leucine, isoleucine, norleucine, valine, norvaline, alanine, 2-aminobutanoic acid, methionine, O-methylserine, t-butylglycine, t-butylalanine, and cyclohexylalanine;

B group: aspartic acid, glutamic acid, isoaspartic acid, isoglutamic acid, 2-aminoadipic acid, and 2-aminosuberic acid;

C group: asparagine and glutamine;

D group: lysine, arginine, ornithine, 2,4-diaminobutanoic acid, and 2,3-diaminopropionic acid;

E group: proline, 3-hydroxy proline, and 4-hydroxy proline;

F group: serine, threonine, and homoserine; and

G group: phenylalanine and tyrosine.

[0113] The transformant of the present invention is not particularly limited as long as the transformant which is obtained by introducing DNA encoding an antibody molecule which specifically binds to human CRTH2 into a host cell and produces the antibody of the present invention. Specific examples thereof include transformants obtained by introducing DNA encoding antibody molecules which specifically binds to human CRTH2 into host cells such as the following (a) to (i).

(a) Chinese hamster ovary tissue-derived CHO cells;

(b) rat myeloma cell line YB2/3HL. P2. G11. 16Ag. 20 cells;

- (c) mouse myeloma cell line NS0 cells;
- (d) mouse myeloma cell line SP2/0-Ag14 cells;
- (e) Syrian hamster kidney tissue-derived BHK cells;
- (f) hybridoma cells producing an antibody;
- (g) human leukemia cell line Namalwa cells;
- (h) embryonic stem cells; and
- (i) amphicytula.

[0114] Further, preferred examples of the transformant of the present invention that produces an antibody in which a N-glycoside-linked sugar chain binds to an Fc region of the antibody and fucose is not bonded to a N-acetyl glucosamine of a reducing terminal of the N-glycoside-linked sugar chain include a transformant obtained by introducing DNA encoding an antibody molecule which specifically binds to human CRTH2 into a host cell in which glycosyltransferase is decreased or deficient which is produced by a method described in Pamphlet of International Publication No. 2005/035586 and Pamphlet of International Publication No.02/31140.

[0115] A method of producing the antibody or the antibody fragment thereof of the present invention is not particularly limited as long as the method is for culturing a transformant that produces the antibody or the antibody fragment thereof of the present invention, and preferred examples thereof include a method of producing an antibody or the antibody fragment thereof by culturing a transformant that produces the antibody or the antibody fragment thereof of the present invention, secreting and accumulating the antibody or the antibody fragment thereof in a culture, and collecting and purifying the antibody or the antibody fragment thereof.

[0116] The antibody or the antibody fragment thereof produced by the above-described production method is also exemplified as the antibody or the antibody fragment thereof of the present invention.

[0117] The composition of the present invention is not particularly limited as long as the composition comprises the antibody or the antibody fragment thereof of the present invention, and examples thereof include a composition comprising antibody molecule in which single type of sugar chain binds thereto and a composition comprising antibody molecules comprising a plurality of sugar chain structures. Further, a composition comprising an appropriate additive and a buffer may be used. Preferred examples of the composition of the present invention include medicine and diagnostic agents comprising the antibody or the antibody fragment thereof of the present invention as an active ingredient.

[0118] The medicine or the diagnostic agent of the present invention is not particularly limited as long as the medicine or the diagnostic agent contains the antibody or the antibody fragment thereof of the present invention as active ingredients. Preferred examples thereof include medicine or diagnostic agent for diseases associated with human CRTH2-expressing cells.

[0119] The treatment method of the present invention is not particularly limited as long as the method is a treatment method of administering an effective amount of antibody or antibody fragment thereof of the present invention, and preferred examples thereof include a method of treating diseases associated with human CRTH2-expressing cells.

[0120] The use of the antibody or the antibody fragment thereof of the present invention is not particularly limited as long as the antibody or the antibody fragment thereof of the present invention is used for producing a therapeutic agent for a disease associated with human CRTH2-expressing cells. Further, the antibody or the antibody fragment thereof of the present invention can be used for at least one of treatment and prevention of a disorder or a disease associated with human CRTH2-expressing cells.

[0121] The disorder or the disease associated with human CRTH2-expressing cells is not particularly limited, and examples thereof include an allergic disease, an autoimmune disease, a disease accompanied by at least one of increase and hyperergasia of eosinophilis, and a disease accompanied by at least one of increase and hyperergasia of Th2 cells and a disease accompanied by at least one of increase and hyperergasia of ILC2.

[0122] Specific examples thereof include allergic or non-allergic rhinitis or sinusitis, chronic sinusitis or rhinitis, nasal polyp, chronic sinusitis accompanied by nasal polyp, eosinophilic sinusitis, acute sinusitis, asthma, childhood asthma, allergic bronchitis, alveolitis, farmer's disease, hypersensitivity respiratory tract, infections, for example, allergic conjunctivitis caused by bacteria, virus, worms, fungi, protozoa, and other pathogens, bronchitis or pneumonitis, bronchiectasis, adult respiratory distress syndrome, bronchus and lung edema, bronchitis, pneumonitis, or interstitial pneumonitis caused by various sources such as poison gas and suction or inhalation of vapor, bronchitis, pneumonitis, or interstitial pneumonitis caused by heart failure, X-ray, radiation, or chemotherapy, bronchitis, pneumonitis, or interstitial pneumonitis associated with collagen disease, for example, erythematosis or generalized scleroderma, pulmonary fibrosis, idiopathic pulmonary fibrosis (IPF), interstitial lung disease or interstitial pneumonitis of various origins, for example, asbestosis, silicosis, m. Boeck, or sarcoidosis, granulomatous disease, cystic fibrosis, mucoviscidosis, α 1-antitrypsin deficiency, eosinophilic cellulite (for example, Wells syndrome), eosinophilic pneumonia (for example, Loeffler syndrome or chronic eosinophilic pneumonia), eosinophilic fasciitis (for example, Shulman syndrome), eosinophilic esophagitis, eosinophils increased syndrome, delayed-type hypersensitivity, non-allergic asthma, exercise-induced bronchoconstriction; chronic obstructive pulmonary disease (COPD), acute bronchitis, chronic bronchitis, emphysema, systemic anaphylaxis, hyper-

sensitivity reaction, drug allergy (for example, with respect to penicillin or cephalosporin), eosinophilia myalgia syndrome caused by ingestion of pollution tryptophan, insect sting allergy; autoimmune disease, for example, rheumatoid arthritis, psoriatic arthritis, multiple sclerosis, systemic lupus erythematosus, myasthenia gravis, immune thrombocytopenia (adult ITP, neonatal thrombocytopenia, pediatric ITP), immune hemolytic anemia (autoimmune and drug-induced), Evans syndrome (platelet and red blood cell immune cytopenia), Rh disease of a newborn, Goodpasture syndrome (anti-GBM disease), celiac disease (Celiac), autoimmune cardiomyopathy, juvenile-onset diabetes; glomerulonephritis, autoimmune thyroiditis, Behcet's disease, graft rejection (for example, in transplantation), for example, homograft rejection or graft-versus-host disease; inflammatory bowel disease, for example, Crohn's disease or ulcerative colitis; spondyloarthropathy; scleroderma; psoriasis (including T-cell mediated psoriasis) and inflammatory skin disease, for example, dermatitis, eczema, atopic dermatitis, allergic contact dermatitis, urticaria (for example, chronic idiopathic, chronic spontaneity, or physical urticaria), bullous pemphigoid; vasculitis (for example, necrotic, cutaneous, granulomatous and hypersensitive angiitis, eosinophilic polyneuropathy vasculitis granulomatous disease); erythema nodosum; eosinophilic myositis, eosinophilic fasciitis, and inflammatory or allergic diseases and conditions including cancer with leukocyte infiltration of the skin or organ.

[0123] Preferred examples of the disorder or the disease associated with human CRTH2-expressing cells include asthma, childhood asthma, chronic obstructive pulmonary disease, atopic dermatitis, allergic rhinitis, and acute or chronic sinusitis.

[0124] The medicine containing the antibody or the antibody fragment thereof of the present invention or a derivative of these may include only the antibody or the antibody fragment thereof or a derivative of these as an effective component, but is typically provided as a medicinal formulation produced by being mixed with one or more carriers pharmacologically acceptable according to a known method in the technical field of pharmaceuticals.

[0125] Examples of administration routes include oral administration and parenteral administration such as oral cavity administration, tracheobronchial administration, rectally administration, subcutaneous administration, intramuscular administration, intravenous administration, or intraperitoneal administration. Examples of administration forms include sprays, capsules, tablets, powders, granules, syrups, emulsions, suppositories, injections, ointments, and tapes.

[0126] Examples of the formulation suitable for oral administration include emulsions, syrups, capsules, tablets, powders, and granules.

[0127] The antibody of the present invention include a derivative of an antibody in which a radioisotope, a small molecule drug, a macromolecule drug, protein, or nucleic acid binds to the antibody or the antibody fragment thereof of the present invention chemically or in a genetic engineering manner.

[0128] In a case where a derivative of an antibody is used as a treatment method, a prophylaxis method, therapeutic agent or therapeutic agent, examples of the drug bound to the antibody or the antibody fragment thereof of the present invention include a chemotherapeutic agent, an antibody drug, an immunostimulant, and a macromolecule drug. Examples of protein include cytokine, growth factors, and toxic protein. Examples of nucleic acid include decoy, antisense, siRNA, and miRNA.

[0129] In a case where a derivative of an antibody is used for a detection method, a quantification method, a reagent for detection, or a reagent for quantification, examples of the drug bound to the antibody or the antibody fragment thereof of the present invention include a marker used for a typical immunological detection method or immunological measurement method.

[0130] In the present invention, a derivative of an antibody can be produced by bonding a radioisotope, a small molecule drug, a macromolecule drug or protein to the N terminal side or the C terminal side of an H chain or an L chain of the antibody or the antibody fragment thereof, an appropriate substituent or a side chain in the antibody or the antibody fragment thereof, or a sugar chain of the antibody or the antibody fragment thereof of the present invention, by a chemical method [methods described in Antibody Engineering Introduction, Chijin Shokan (1994) or the like].

[0131] In the present invention, a derivative of an antibody can be produced by a genetic engineering method wherein DNA encoding the antibody or the antibody fragment thereof of the present invention is connected with DNA encoding protein intended to be connected, the DNA is inserted into an expression vector, and the expression vector is introduced into an appropriate host cell to express the derivative.

[0132] Examples of the radioisotope include ^{131}I , ^{125}I , ^{90}Y , ^{64}Cu , ^{99}Tc , ^{77}Lu , and ^{211}At . The radioisotope can be directly bonded to an antibody by a chloramine-T method or the like. Further, a substance that chelates the radioisotope may be bonded to an antibody. Examples of the chelating agent include 1-isothiocyanatobenzyl-3-methyldiethylenetriamine pentaacetate (MX-DTPA).

[0133] Examples of the small molecule drug include a light emitting substance such as acridinium ester or lophine and a fluorescent substance such as fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (RITC).

[0134] Examples of the method of bonding a small molecule drug to an antibody include a method of bonding a chemical agent to an amino group of an antibody using glutaraldehyde and a method of bonding an amino group of a chemical agent to a carboxyl group of an antibody using water-soluble carbodiimide.

[0135] Examples of the macromolecule drug include polyethylene glycol (hereinafter, referred to as PEG), albumin,

dextran, polyoxyethylene, a styrene-maleic acid copolymer, polyvinylpyrrolidone, a pyran copolymer, and hydroxypropyl methacrylamide.

[0136] The immunological detection method or the immunological measurement method is a method of detecting or measuring the amount of antibody or the amount of antigen using the antigen or antibody which is labeled. Examples of the immunological detection method or the immunological measurement method include a radioactive substance-labeled immunoantibody assay (RIA) method, an enzyme immunoassay (EIA or ELISA) method, a fluorescent immunoassay (FIA) method, a luminescent immunoassay method, a western blotting method, and a physico-chemical method.

[0137] Diseases associated with human CRTH2 can be diagnosed by detecting or measuring human CRTH2-expressing cells according to the above-described method using the antibody or the antibody fragment thereof of the present invention.

[0138] A biological sample which is a subject for detecting or measuring human CRTH2 of the present invention is not particularly limited as long as the sample may include human CRTH2 or a peptide fragment having a part thereof secreted out of cells, or human CRTH2-expressing cells, such as tissue cells, blood, plasma, serum, pancreatic juice, urine, feces, tissue fluid, bronchoalveolar lavage, or a culture solution.

[0139] The diagnostic agent containing the antibody or the antibody fragment thereof of the present invention or a derivative of these may include a reagent for carrying out an antigen-antibody reaction or a reagent for detecting the reaction according to a diagnostic method. Examples of the reagent for carrying out an antigen-antibody reaction include a buffering agent and salts. Examples of the reagent for detecting the reaction include reagents used for a typical immunological detection method or immunological measurement method, such as a secondary antibody labeled which recognizes the antibody, the antibody fragment thereof or a derivative of these, or a substrate corresponding to the label.

[0140] Hereinafter, a method of producing the antibody of the present invention, a method of treating a disease, and a method of diagnosing a disease will be described in detail.

1. Method of producing antibody

(1) Preparation of antigen

[0141] Human CRTH2 as an antigen or cells expressing human CRTH2 can be obtained by introducing an expression vector comprising cDNA that encodes the full length or the partial length of human CRTH2 into *E. coli*, yeast, insect cells, or animal cells.

[0142] Further, the antigen can be also obtained by purifying human CRTH2 from various human cultured cells or human tissues or the like which express a large amount of human CRTH2. Moreover, the cultured cells or the tissues can be used as an antigen as they are. Further, a synthetic peptide comprising a partial sequence of human CRTH2 is prepared by a chemical synthetic method such as an Fmoc method or a tBoc method and then can be used as an antigen.

[0143] A known tag such as FLAG or His may be added to the C terminal or the N terminal of the synthetic peptide comprising human CRTH2 or a partial sequence of human CRTH2.

[0144] The human CRTH2 used in the present invention can be produced by expressing DNA encoding the human CRTH2 in a host cell using a method described in *Molecular Cloning, A Laboratory Manual, Second Edition*, Cold Spring Harbor Laboratory Press (1989) or *Current Protocols in Molecular Biology*, John Wiley & Sons (1987 to 1997), for example, the following method.

[0145] First, a recombinant vector is prepared by inserting full-length cDNA comprising a part that encodes human CRTH2 into the downstream of a promoter of an appropriate expression vector. A DNA with suitable length fragment which is prepared based on the full-length cDNA, has a part that encodes a polypeptide may be used in place of the above described full-length cDNA. Next, a transformant that produces the polypeptide can be obtained by introducing the recombinant vector to be obtained into a host cell suitable for the expression vector.

[0146] The expression vector is not particularly limited as long as the expression vector can be autonomously replicated or integrated into a chromosome in a host cell being used and includes a suitable promoter at a position where DNA encoding a polypeptide can be transcribed.

[0147] The host cell is not particularly limited as long as the host cell can express a target gene and the example thereof is a microorganism belonging to the genus *Escherichia* such as *E. coli*, yeast, insect cells, or animal cells.

[0148] In a case where a prokaryote such as *E. coli* is used as a host cell, it is preferable that the recombinant vector enables autonomous replication in a prokaryote and includes a promoter, a ribosome-binding sequence, DNA comprising a part that encodes human CRTH2, and a transcription termination sequence.

[0149] Moreover, the recombinant vector does not necessarily have a transcription termination sequence, but it is preferable that a transcription termination sequence is arranged immediately right after a structural gene. Further, the recombinant vector may include genes controlling a promoter.

[0150] As the recombinant vector, it is preferable to use a plasmid in which the distance between a Shine-Dalgarno sequence (also referred to as an SD sequence) which is a ribosome-binding sequence and initiation codon is appropriately

adjusted (for example, 6 to 18 bases).

[0151] Moreover, in a base sequence of DNA encoding the human CRTH2, a base can be substituted so as to be a codon optimal for expression in a host and thus the production rate of desired human CRTH2 can be improved.

[0152] The expression vector is not particularly limited as long as the expression vector is capable of exhibiting the function in a host cell being used, and examples thereof include pBTrp2, pBTac1, pBTac2 (all Roche Diagnostics K.K.), pKK233-2 (Pharmacia Corp.), pSE280 (Invitrogen), pGEMEX-1 (Promega Corporation), pQE-8 (QIAGEN), pKYP10 (JP-A-S58-110600), pKYP200 [Agricultural Biological Chemistry, 48,669(1984)], pLSA1 [Agric Biol. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad. Sci. USA, 82, 4306 (1985)], pBluescript II SK (-) (Stratagene), pTrs30 [prepared from E. coli JM109/pTrs30 (FERM BP-5407)], pTrs32 [prepared from E. coli JM109/pTrs32 (FERM BP-5408)], pGHA2 [prepared from E. coli IGHA2 (FERM BP-400), JP-A-S60-221091], pGKA2 [prepared from E. coli IGKA2 (FERM BP6798), JP-A-S60-221091], pTerm2 (US Patent No. 4686191, US Patent No. 4939094, and US Patent No. 5160735), pSupex, pUB110, pTP5, pC194, pEG400 [J. Bacteriol., 172, 2392 (1990)], pGEX (Pharmacia Corp.), pET system (Novagen), and pME18SFL3.

[0153] The promotor is not particularly limited as long as the promotor can exhibit the function in a host cell being used. Examples thereof include promotors derived from E. coli or phage such as a trp promotor (Ptrp), a lac promotor, a PL promotor, a PR promotor, and a T7 promotor. Further, promotors whose designs are artificially changed, such as a tandem promotor in which two Ptrps are serially arranged, a tac promotor, a lacT7 promotor, and a letI promotor can be used.

[0154] Examples of the host cell include E. coli XL-1Blue, E. coli XL2-Blue, E. coli DH1, E. coli MC1000, E. coli KY3276, E. coli W1485, E. coli JM109, E. coli HB101, E. coli No. 49, E. coli W3110, E. coli NY49, and E. coli DH5 α .

[0155] The method of introducing a recombinant vector into a host cell is not particularly limited as long as the method is for introducing DNA into a host cell being used, and examples thereof include a method of using calcium ions [Proc. Natl. Acad. Sci. USA, 69, 2110 (1972); Gene, 17, 107 (1982); Molecular & General Genetics, 168, 111 (1979)].

[0156] In a case where animal cells are used as host cells, the expression vector is not particularly limited as long as the vector can exhibit the function in animal cells and examples thereof include pcDNA1, pcDM8 (Funakoshi Co., Ltd.), pAGE107 [JP-A-H03-22979; Cytotechnology, 3, 133 (1990)], pAS3-3 (JP-A-H02-227075), pcDM8 [Nature, 329, 840 (1987)], pcDNA I/Amp (Invitrogen), pcDNA 3.1 (Invitrogen), pREP4 (Invitrogen), pAGE103 [J. Biochemistry, 101, 1307 (1987)], pAGE210, pME18SFL3, pKANTEX93 (Pamphlet of International Publication No. 97/10354), N5KG1val (US Patent No. 6001358), INPEP4 (Biogen-IDEC), and a transposon vector (Pamphlet of International Publication No. 2010/143698).

[0157] The promotor is not particularly limited as long as the promotor can exhibit the function in animal cells, and examples thereof include a promotor of immediate early (IE) genes of cytomegalovirus (CMV), an early promotor of SV40, a promotor of retrovirus, a metallothionein promotor, a heat shock promotor, an SR α promotor, and a promotor or an enhancer of Moloney mouse leukemia virus. Further, an enhancer of IE genes of human CMV may be used with a promotor.

[0158] Examples of host cells include human leukemia cell Namalwa cells, monkey cell COS cells, Chinese hamster ovary cell CHO cells [Journal of Experimental Medicine, 108, 945 (1958); Proc. Natl. Acad. Sci. USA, 60, 1275 (1968); Genetics, 55, 513 (1968); Chromosoma, 41, 129 (1973); Methods in Cell Science, 18, 115 (1996); Radiation Research, 148, 260 (1997); Proc. Natl. Acad. Sci. USA, 77, 4216 (1980); Proc. Natl. Acad. Sci. USA, 60, 1275 (1968); Cell, 6, 121 (1975); Molecular Cell Genetics, Appendix I, II (pp. 883 to 900)], CHO/DG44, CHO-K1 (ATCC No: CCL-61), DUKXB11 (ATCC No: CCL-9096), Pro-5 (ATCC No: CCL-1781), CHO-S (Life Technologies, Cat #11619), Pro-3, rat myeloma cells YB2/3HL. P2. G11. 16Ag. 20 (or referred to as YB2/0), mouse myeloma cells NS0, mouse myeloma cells SP2/0-Ag14, and Syrian hamster cells BHK or HBT5637 (JP-A-S63-000299).

[0159] The method of introducing a recombinant vector into a host cell is not particularly limited as long as the method is for introducing DNA into an animal cell, and examples thereof include an electroporation method [Cytotechnology, 3, 133 (1990)], a calcium phosphate method (JP-A-H02-227075), and a lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)].

[0160] Human CRTH2 can be produced by culturing a transformant derived from a microorganism or an animal cell that includes a recombinant vector in which DNA encoding human CRTH2 is integrated obtained in the above-described manner in a culture medium, secreting and accumulating the human CRTH2 in a culture, and collecting the human CRTH2 from the culture. The method of culturing the transformant in a culture medium can be carried out according to a typical method used for culturing a host.

[0161] In a case where cells derived from a eukaryote are used for expression, human CRTH2 to which sugar or a sugar chain is added can be obtained.

[0162] When a microorganism transformed by a recombinant vector using an inductive promotor is cultured, an inducer may be added to a culture medium if necessary. For example, isopropyl- β -D-thiogalactopyranoside may be added to a culture medium in which a microorganism transformed by a recombinant vector using a lac promotor is cultured and indole acrylic acid may be added to a culture medium in which a microorganism transformed by a recombinant

vector using a trp promotor is cultured.

[0163] Examples of the culture medium for culturing a transformant obtained from animal cells as a host include generally used RPMI1640 culture medium [The Journal of the American Medical Association, 199, 519 (1967)], an Eagle's MEM culture medium [Science, 122, 501 (1952)], Dubecco's modified MEM culture medium [Virology, 8, 396 (1959)], 199 culture medium [Proc. Soc. Exp. Biol. Med., 73, 1 (1950)], Iscove's Modified Dulbecco's medium (IMDM) culture medium, and culture media obtained by adding fetal bovine serum (FBS) or the like to these culture media. The culturing is usually carried out under the conditions of pH of 6 to 8 at 30°C to 40°C for 1 to 7 days in the presence of 5% CO₂. Further, antibiotics such as kanamycin or penicillin may be added to a culture medium during the culturing if necessary.

[0164] As a method of expressing genes encoding human CRTH2, a method of secretory production or fusion protein expression [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)] can be used in addition to direct expression.

[0165] Examples of the method of producing human CRTH2 include a method of producing human CRTH2 in a host cell, a method of secreting human CRTH2 out of a host cell, and a method of producing human CRTH2 on an outer membrane of a host cell, and an appropriate method can be selected by changing the host cell being used or the structure of human CRTH2 to be produced.

[0166] In a case where human CRTH2 is produced in a host cell or on an outer membrane of a host cell, human CRTH2 can be actively secreted outside the host cell using a method of Paulson et al. [J. Biol. Chem., 264, 17619 (1989)], a method of Lowe et al. [Proc. Natl. Acad. Sci. USA, 86, 8227 (1989); Genes Develop., 4, 1288 (1990)], and methods described in JP-A-H05-336963) and Pamphlet of International Publication No. 94/23021.

[0167] In addition, the amount of producing human CRTH2 can be increased with a gene amplification system (JP-A-H02-227075) using dihydroforate reductase genes.

[0168] The human CRTH2 to be obtained can be isolated or purified in the following manner for example. In a case where human CRTH2 in cells is expressed as a soluble protein, cells are recovered by centrifugation after culturing and disrupted using an ultrasonic crusher, a French press, a Manton Gaulin homogenizer, or a dynamill after suspension in an aqueous buffer, thereby obtaining a cell-free extract.

[0169] A purified preparation can be obtained from a supernatant obtained by centrifugation of the cell-free extract is centrifuged, using a typical method of isolating and purifying protein, that is, a solvent extraction method, a salting-out method using ammonium sulfate and the like, a desalting method, a precipitation method using an organic solvent, anion exchange chromatography using a resin such as diethylaminoethyl (DEAE)-Sepharose or DIAION HPA-75 (Mitsubishi Chemical Corporation), cation exchange chromatography using a resin such as S-Sepharose FF (Pharmacia Corp.), hydrophobic chromatography using a resin such as butyl Sepharose or phenyl Sepharose, gel filtration using a molecular sieve, affinity chromatography, a chromatofocusing method, or an electrophoresis method such as isoelectric point electrophoresis. These methods may be used alone or in combination.

[0170] In a case where human CRTH2 is expressed as an insoluble matter in cells, the insoluble matter of the human CRTH2 are recovered as a precipitate fraction by disrupting the cells in the above-described manner after recovering and performing centrifugation. The recovered insoluble matter of the human CRTH2 is solubilized by a protein denaturing agent. A purified preparation of a polypeptide can be obtained by diluting or dialyzing the solubilized solution after the human CRTH2 is restored to have a normal three-dimensional structure using the same isolation and purification method as described above.

[0171] In a case where human CRTH2 or the derivative thereof such as glycosylated product is secreted outside the cells, the derivative of human CRTH2 or a sugar modification can be recovered from a culture supernatant. A soluble fraction is obtained by treating the culture through a method of centrifugation or the like in the same manner as described above and the same isolation and purification method as described above is used, thereby obtaining a purified preparation from the soluble fraction.

[0172] Human CRTH2 used in the present invention can be produced by a chemical synthesis method such as an Fmoc method or a tBoc method. Further, human CRTH2 can be chemically synthesized using a peptide synthesizer of Advanced Chem Tech, PerkinElmer Inc., Pharmacia Corp., Protein Technology Instruments, Synthecell-Vega Corp., Perceptive Inc., Shimadzu Corporation or the like.

(2) Immunization of animal and preparation of antibody-producing cells for fusion

[0173] An animal such as a mouse, rat, or hamster which is 3- to 20-week-old is immunized with the antigen obtained in (1) described above and antibody-producing cells in the spleen, the lymph node, and the peripheral blood of the animal is collected. Further, in a case where immunogenicity is low and the antibody titer is not sufficiently increased in the animal, human CRTH2 knockout mouse can be used as an animal to be immunized.

[0174] An animal is immunized by subcutaneous administration, tail administration, intravenous administration, or intraperitoneal administration of an antigen together with an appropriate adjuvant such as Freund's complete adjuvant

or aluminum hydroxide gel or pertussis vaccine. In a case where the antigen is a partial peptide, conjugate are prepared with carrier protein such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) and these are used immunogens.

[0175] An antigen is administered 1 to 10 times at one- to two-week interval after first administration. The blood is collected from venous plexus of eye fundus or tail vein at the third to seventh day after each administration and the antibody titer of the serum is measured using an enzyme immunoassay method [Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)] or the like. An animal whose serum shows sufficient antibody titer with respect to an antigen used for immunization is used as a supply source of antibody-producing cells for fusion.

[0176] At the third to seventh day after final administration of an antigen, tissues including antibody-producing cells of the immunized animal, such as the spleen or the like, are resected and antibody-producing cells are collected. In a case of using spleen cells, the spleen is cut, loosened, and centrifuged and red blood cells are removed, thereby obtaining antibody-producing cells for fusion.

(3) Preparation of myeloma cells

[0177] As myeloma cells, established cells obtained from a mouse, for example, 8-azaguanine-resistant mouse (BALB/c-derived) myeloma cell line P3-X63Ag8-U1 (P3-U1) [Current Topics in Microbiology and Immunology, 18, 1 (1978)], P3-NS1/1Ag41 (NS-1) [European J. Immunology, 6, 511 (1976)], SP2/0-Ag14 (SP-2) [Nature, 276, 269 (1978)], P3-X63-Ag8653 (653) [J. Immunology, 123, 1548 (1979)], or P3-X63-Ag8 (X63) [Nature, 256, 495 (1975)] are used.

[0178] The myeloma cells are subcultured in a normal culture medium [RPMI1640 culture medium to which glutamine, 2-mercaptoethanol, gentamicin, FBS, and 8-azaguanine are added] and subcultured in a normal culture medium before 3 to 4 days of cell fusion to ensure 2×10^7 or more cells on the day of fusion.

(4) Cell fusion and preparation of monoclonal antibody-producing hybridomas

[0179] The antibody-producing cells for fusion obtained in (2) described above and the myeloma cells obtained in (3) described above are washed with a minimum essential medium (MEM) culture medium or PBS (1.83 g of disodium phosphate, 0.21 g of monopotassium phosphate, 7.65 g of salt, 1 L of distilled water, pH of 7.2) mixed with each other so as to yield the ratio of the cell number of antibody-producing cells for fusion : myeloma cells = 5:1 to 10:1, and the supernatant is removed after centrifugation.

[0180] After the precipitated cell group is sufficiently loosened, a mixed solution of polyethylene glycol-1000 (PEG-1000), MEM culture medium, and dimethyl sulfoxide is added while being stirred at 37°C. Further, 1 to 2 mL of the MEM culture medium is added several times at 1- to 2-minute interval, and then MEM culture medium is added to yield a total amount of 50 mL. After centrifugation, the supernatant is removed.

[0181] After the precipitated cell group is gently loosened, antibody-producing cells for fusion are gently suspended in HAT culture medium [normal culture medium to which hypoxanthine, thymidine, and aminopterin are added]. The suspension is cultured in a 5% CO₂ incubator at 37°C for 7 to 14 days.

[0182] Apart of the culture supernatant is sampled after culturing, a cell group which reacts with an antigen including human CRTH2 and does not react with an antigen that does not include human CRTH2 is selected according to a method of selecting hybridomas, such as reactivity analysis against human CRTH2-expressing cells described below. Next, cloning is repeated twice by a limiting dilution method [an HT culture medium (culture medium obtained by removing aminopterin from an HAT culture medium) is used for the first cloning and a normal culture medium is used for the second cloning] and then hybridomas recognized to stably have strong antibody titer are selected as monoclonal antibody-producing hybridomas.

(5) Preparation of purified monoclonal antibody

[0183] The monoclonal antibody-producing hybridomas obtained in (4) described above are intraperitoneally injected to a Pristine-treated [0.5 mL of 2,6,10,14-tetramethylpentadecane (Pristane) is intraperitoneally administered and the animal is bred for 2 weeks] 8- to 10-week-old mouse or nude mouse. The hybridomas develop ascites cancer after 10 to 21 days. After the ascites fluid is collected from the mouse and the solid content is removed by centrifugation, the fluid is subject to salting out with 40 to 50% ammonium sulfate, purification is carried out using a caprylic acid precipitation method, a DEAE-sepharose column, a protein A-column, or a gel filtration column, and then IgG fractions or IgM fractions are collected to obtain a purified monoclonal antibody.

[0184] Furthermore, after the monoclonal antibody-producing hybridomas obtained in (4) described above are cultured in an RPMI1640 culture medium to which 10% FBS is added, the supernatant is removed by centrifugation, suspended in a Hybridoma-SFM culture medium, and cultured for 3 to 7 days. The obtained cell suspension is centrifuged, the obtained supernatant is purified using a protein A-column or a protein G-column, and IgG fractions are collected, thereby

obtaining a purified monoclonal antibody. Further, 5% Daigo's GF21 can be added to the Hybridoma-SFM culture medium.

[0185] The subclass of the antibody is determined by an enzyme immunoassay method using a subclass typing kit. The amount of protein is calculated by a Lowry method or using absorbance at 280 nm.

(6) Selection of monoclonal antibody

[0186] A monoclonal antibody is selected by analyzing the reactivity against human CRTH2-expressing cells using flow cytometry as described below.

[0187] Examples of the human CRTH2-expressing cells include transgenic cells obtained by introducing an expression vector comprising cDNA encoding human CRTH2 obtained in (1) described above into animal cells or the like, human eosinophils, basophils, Th2, ILC2, nonclassical monocyte, and Th2/Th17 cells.

[0188] After the cells are dispensed to a plate such as a 96 well plate, the substance to be tested such as the serum, a culture supernatant of hybridomas, or a purified monoclonal antibody as a first antibody is dispensed for reaction. The cells after the reaction are washed with PBS including 1% to 10% of bovine serum albumin (BSA) (hereinafter, referred to as BSA-PBS) or the like, and then an anti-immunoglobulin antibody labeled with a fluorescent reagent as a second antibody is dispensed for reaction. After the cells are washed with BSA-PBS or the like, a monoclonal antibody specifically reacting against expressing cells is selected by measuring the amount of fluorescence of the labeled antibody using flow cytometer.

[0189] In addition, a monoclonal antibody that competes the monoclonal antibody of the present invention in the binding to human CRTH2 can be obtained by adding a test antibody to a binding reaction-detecting system using the above-described flow cytometry to cause a reaction.

[0190] That is, a monoclonal antibody that competes the monoclonal antibody obtained in the present invention in the binding to an amino acid sequence of human CRTH2 or the three-dimensional structure thereof, can be obtained by screening an antibody in which the binding of the monoclonal antibody of the present invention is inhibited at the time when the test antibody is added.

[0191] An antibody which binds to the same epitope as the epitope recognized by the monoclonal antibody of the present invention which binds to the amino acid sequence or the three-dimensional structure of human CRTH2 can be obtained by identifying the epitope of the antibody obtained by the binding reaction-detecting system using the above-described flow cytometry and preparing a partial synthetic peptide of the identified epitope or a synthetic peptide mimicking the three-dimensional structure of the epitope and immunize with the peptide.

2. Preparation of recombinant antibody

[0192] As an example of preparing a recombinant antibody, a method of preparing a human chimeric antibody and a humanized antibody will be described below.

(1) Construction of recombinant antibody expression vector

[0193] A recombinant antibody expression vector is an expression vector for animal cell in which DNA encoding CH and CL of a human antibody is incorporated and can be constructed by cloning DNA encoding the CH and the CL of the human antibody to the expression vector for animal cell.

[0194] As C region of a human antibody CH and CL of any human antibody may be used. For example, CH of a γ 1 subclass and CL of a κ class of a human antibody are used. cDNA is used as DNA encoding CH and CL of a human antibody, but chromosome DNA formed of an exon and an intron can be used.

[0195] The expression vector for animal cell is not particularly limited as long as a vector in which genes encoding the C region of a human antibody are incorporated and expressed. For example, pAGE107 [Cytotechnol., 3, 133 (1990)], pAGE103 [J. Biochem., 101, 1307 (1987)], pHSG274 [Gene, 27, 223 (1984)], pKCR [Proc. Natl. Acad. Sci. USA, 78, 1527 (1981)], pSGlbd2-4 [Cytotechnol., 4, 173 (1990)], pSE1UK1Sed1-3 [Cytotechnol., 13, 79 (1993)] or the like is used.

[0196] As a promotor and an enhancer used for an expression vector for animal cell, an initial promo promotor of SV40 [J. Biochem., 101, 1307 (1987)], Moloney mouse leukemia virus LTR [Biochem. Biophys. Res. Commun., 149, 960 (1987)], a promotor of the immunoglobulin H chain [Cell, 41, 479 (1985)] or the like, and an enhancer [Cell, 33, 717 (1983)] are used.

[0197] From the viewpoint that a recombinant antibody expression vector is easily constructed and easily introduced into animal cells and the amount of expressing an antibody H chain and the amount of expressing an antibody L chain in animal cells are balanced, a tandem type recombinant antibody expression vector [J. Immunol. Methods, 167, 271 (1994)] in which the antibody H chain and the antibody L chain are present on the same vector is used as the recombinant antibody expression vector, but a separator vector in which the antibody H chain and the antibody L chain are present on vectors different from each other can be used. pKANTEX93 (Pamphlet of International Publication No. 97/10354) or

pEE18 [Hybridoma, 17, 559 (1998)] is used as the tandem type recombinant antibody expression vector.

(2) Obtaining of cDNA encoding V region of antibody derived from non-human animal and analysis of amino acid sequence

[0198] Obtaining of cDNA encoding VH and VL of a non-human antibody and analysis of the amino acid sequence can be performed in the following manner.

[0199] mRNA is extracted from hybridoma cells producing a non-human antibody and cDNA is synthesized. The synthesized cDNA is cloned to a vector such as a phage or a plasmid, thereby preparing a cDNA library.

[0200] A recombinant phage or a recombinant plasmid comprising cDNA encoding VH or VL is isolated from the above described library using DNA encoding a C region portion or a V region portion of a mouse antibody as a probe. The entire base sequences of VH or VL of a mouse antibody of interest on the recombinant phage or recombinant plasmid are respectively determined and the entire amino acid sequences of VH or VL are respectively estimated by the base sequences.

[0201] The non-human animal for preparing hybridoma cells producing a non-human antibody, a mouse, a rat, a hamster, or a rabbit may be used, but the animal is not particularly limited as long as hybridoma cells can be prepared.

[0202] A guanidine thiocyanate-caesium trifluoroacetate method [Methods in Enzymol., 154, 3 (1987)] or a kit such as RNA easy kit (QIAGEN) is used for preparation of total RNA from hybridoma cells.

[0203] An oligo (dT) immobilized cellulose column method [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)] or a kit such as an Oligo-dT30<Super>mRNA Purification Kit (TAKARA BIO INC.) is used for preparation of mRNA from total RNA. Further, mRNA can be prepared from hybridoma cells using a kit such as a Fast Track mRNA Isolation Kit (Invitrogen) or a QuickPrep mRNA Purification Kit (Pharmacia).

[0204] A known method [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989), Current Protocols in molecular Biology, Supplement 1, John Wiley & Sons (1987 to 1997)], a SperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (Invitrogen), or a kit such as a ZAP-cDNA Synthesis Kit (Stratagene) is used for synthesis of cDNA and preparation of cDNA library.

[0205] When cDNA library is prepared, the vector in which cDNA obtained by synthesis using mRNA extracted from hybridoma cells as a template is incorporated is not particularly limited as long as the cDNA can be incorporated in the vector.

[0206] For example, ZAP Express [Strategies, 5,58 (1992)], pBluescript II SK (+) [Nucleic Acids Research, 17, 9494 (1989)], λ ZAPII (Stratagene), λ gt10, λ gt11 [DNA Cloning: A Practical Approach, I,49 (1985)], Lambda BlueMid (Clontech Laboratories, Inc.), λ Ex Cell, pT7T3-18U (Pharmacia), pcD2 [Mol. Cell. Biol., 3,280 (1983)], or pUC18 [Gene, 33, 103 (1985)] is used.

[0207] E. coli into which cDNA library to be constructed by a phage or a plasmid vector is introduced is not particularly limited as long as the cDNA library can be introduced, expressed, and maintained. For example, XL-1Blue MRF [Strategies, 5,81 (1992)], C600 [Genetics, 39,440 (1954)], Y1088, Y1090 [Science, 222, 778 (1983)], NM522 [J. Mol. Biol., 166, 1 (1983)], K802 [J. Mol. Biol., 16, 118 (1966)], or JM105 [Gene, 38,275 (1985)] is used.

[0208] A colony hybridization method, a plaque hybridization method using an isotope or a fluorescence-labeled probe [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)] or the like is used for selection of a cDNA clone encoding VH or VL of a non-human antibody from the cDNA library.

[0209] Further, cDNA encoding VH or VL can be prepared by preparing a primer and performing a Polymerase Chain Reaction method [hereinafter, referred to as a PCR method, Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989), Current Protocols in molecular Biology, Supplement 1, John Wiley & Sons (1987 to 1997)] using cDNA or cDNA library synthesized from mRNA as a template.

[0210] After selected cDNA is cut by an appropriate restriction enzyme or the like, the fragment is cloned to a plasmid such as pBluescript SK(-) (Stratagene) and the base sequence of the cDNA is determined by a base sequence analysis method being typically used. The base sequence analysis method is carried out using a base sequence automatic analyzer such as ABI PRISM 3700 (PE Biosystems) or A. L. F. DNA sequencer (Pharmacia) after the reaction of a dideoxy method [Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)] or the like.

[0211] The entire amino acid sequences of VH and VL are respectively deducted from the determined base sequence and it is confirmed whether the obtained cDNA encodes the complete amino acid sequences of VH and VL of an antibody comprising the secretory signal sequence by comparing with the entire amino acid sequences [A. L. F. DNA, US Dept. Health and Human Services (1991)] of VH and VL of a known antibody.

[0212] In regard to the complete amino acid sequences of VH and VL of an antibody including the secretory signal sequence, the length of the secretory signal sequence and the N terminal amino acid sequence can be estimated by comparing the complete amino acid sequences of VH and VL of a known antibody [A. L. F. DNA, US Dept. Health and Human Services (1991)] and the sub-group to which they belong can be known. Further, amino acid sequences of each CDR of VH and VL can be also found by comparing the amino acid sequences of VH and VL of a known antibody [A. L. F. DNA, US Dept. Health and Human Services (1991)].

[0213] It is possible to confirm whether the complete amino acid sequences of VH and VL are new by performing homology search such as a BLAST method [J. Mol. Biol., 215, 403 (1990)] in any database such as SWISS-PROT or PIR-Protein using the complete amino acid sequences of VH and VL to be obtained.

5 (3) Construction of human chimeric antibody expression vector

[0214] A human chimeric antibody expression vector can be constructed by cloning cDNA encoding VH and VL of a non-human antibody to the upstream of each gene encoding CH or CL of a human antibody of the recombinant antibody expression vector obtained in (1) described above.

10 **[0215]** In order to connect the 5' terminal side of CH or CL of a human antibody to the 3' terminal side of cDNA encoding VH or VL of a non-human antibody, cDNA encoding VH and VL is prepared so as to encode appropriate amino acids and have an appropriate recognition sequence of a restriction enzyme at a linkage position.

[0216] A human chimeric antibody expression vector is constructed by cloning the prepared cDNA of VH and VL to the upstream of each gene encoding CH and CL of a human antibody of a humanized antibody expression vector
15 obtained in (1) described above so as to be expressed in an appropriate form.

[0217] Further, cDNA encoding VH or VL of a non-human antibody is amplified by a PCR method using synthetic DNA comprising recognition sequences of an appropriate restriction enzyme at both ends and can be cloned to the recombinant antibody expression vector obtained in (1) described above.

20 (4) Construction of cDNA encoding V region of humanized antibody

[0218] cDNA encoding VH and VL of a humanized antibody can be constructed in the following manner.

[0219] Amino acid sequences of FR of VH or VL of a human antibody for transplanting amino acid sequences of CDR of VH or VL of a non-human antibody are respectively selected. The amino acid sequence of FR to be selected is not
25 particularly limited as long as the amino acid sequence is derived from a human antibody.

[0220] For example, the amino acid sequences of FR of a human antibody registered in the database such as Protein Data Bank or common amino acid sequences in each sub-group of FR of a human antibody [A.L.F. DNA, US Dept. Health and Human Services (1991)] or the like are used. In order to suppress decrease of binding activity of an antibody, an amino acid sequence of FR having homology (at least 60%) as high as possible with the amino acid sequence of FR
30 of VH or VL of an original antibody is selected.

[0221] Next, the amino acid sequences of CDR of the original antibody are transplanted to the amino acid sequences of selected FR of VH or VL of the human antibody, respectively to design the amino acid sequences of VH or VL of a humanized antibody. The designed amino acid sequences are converted to DNA sequences in consideration of the frequency of use of codon shown in the base sequences of genes of the antibody [A.L.F. DNA, US Dept. Health and
35 Human Services (1991)] to design the DNA sequences encoding the amino acid sequences of VH or VL of the humanized antibody are respectively designed.

[0222] Based on the designed DNA sequences, several strands of synthetic DNA comprising the length of approximately 100 bases are synthesized and PCR reaction is carried out using this synthetic DNA. In this case, based on the reaction efficiency of the PCR reaction and the length of DNA which can be synthesized, six strands of synthetic DNA
40 are respectively designed preferably for the H chain and the L chain. Further, cDNA encoding VH or VL of a humanized antibody can be easily cloned to the humanized antibody expression vector obtained in (1) described above by introducing the recognition sequence of an appropriate restriction enzyme into the 5' terminal of synthetic DNA positioned at both ends.

[0223] After the PCR reaction, each of the amplified product is cloned to plasmids such as pBluescript SK (-) (Stratagene), the base sequence is determined in the same manner as the method described in (2), and then plasmids
45 comprising DNA sequences encoding the amino acid sequences of the entire H chain and the entire L chain of a desired humanized antibody are obtained.

[0224] Alternatively, based on the designed DNA sequences, the full-length VH and the full-length VL which are respectively synthesized as one strand of long-chain DNA can be used in place of the above described PCR amplified product. Further, cDNA encoding VH or VL of a humanized antibody can be easily cloned to the humanized antibody
50 expression vector obtained in (1) described above by introducing the recognition sequence of an appropriate restriction enzyme into both ends of the synthetic long-chain DNA.

(5) Modification of amino acid sequence in V region of humanized antibody

55 **[0225]** When only CDR of VH and VL of a non-human antibody is transplanted to FR of VH and VL of a human antibody, the antigen-binding activity of the humanized antibody decreases compared to that of an original non-human antibody [BIO/TECHNOLOGY, 9, 266 (1991)]. In a humanized antibody, antigen-binding activity which has decreased can be improved by identifying amino acid residues involved in direct binding to an antigen, amino acid sequences interacting

with the amino acid residues of CDR and amino acids which maintain the three-dimensional structure of the antibody and thereby involved in indirect binding to an antigen among the amino acid sequences of FR of VH and VL of a human antibody, and substituting these amino acid residues with amino acid residues of an original non-human antibody.

[0226] By using X-ray crystallographic analysis [J. Mol. Biol., 112, 535 (1977)] or computer modeling [Protein Engineering, 7, 1501 (1994)] or the like to identify amino acid residues of FR associated with the antigen-binding activity, construction and analysis of the three-dimensional structure of an antibody can be performed. Further, when multiple variants are prepared for respective antibodies and the correlation between the variants and the antigen-binding activities thereof is repeatedly examined, a humanized antibody having necessary antigen-binding activity can be obtained after trial and error.

[0227] The amino acid residue of FR of VH and VL of a human antibody can be substituted by carrying out the PCR reaction described in (4) using synthetic DNA for modification. Regarding the amplified product after the PCR reaction, the base sequence is determined by the method described in (2) and desired modification which has been carried out is confirmed.

(6) Construction of humanized antibody expression vector

[0228] A humanized antibody expression vector can be constructed by cloning cDNA encoding VH or VL of the constructed recombinant antibody to the upstream of gene encoding CH or CL of a human antibody in the gene recombinant expression vector obtained in (1) described above respectively.

[0229] For example, among the synthetic DNA used for constructing VH or VL of the humanized antibody obtained in (4) and (5) described above, a recognition sequence of an appropriate restriction enzyme is introduced into the 5' terminal of synthetic DNA positioned at both ends, whereby DNA is cloned to the upstream of each gene encoding CH or CL of a human antibody of the humanized antibody expression vector obtained in (1) described above so as to be expressed in an appropriate form.

(7) Transient expression of recombinant antibody

[0230] The antigen-binding activity of plural kinds of prepared gene recombinant antibodies can be efficiently evaluated by performing transient expression of a recombinant antibody using the recombinant antibody expression vectors obtained in (3) and (6) described above or expression vectors obtained by modifying the recombinant antibody expression vectors.

[0231] The host cell into which an expression vector is introduced is not particularly limited as long as the host cell is capable of expressing a recombinant antibody, and for example COS-7 cells (ATCC No: CRL1651) are used [Methods in Nucleic Acids Res., CRC Press, 283 (1991)].

[0232] An expression vector is introduced into COS-7 cells using a DEAE-dextran method [Methods in Nucleic Acids Res., CRC Press (1991)], a lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)] or the like.

[0233] After an expression vector is introduced, the expression amount and the antigen-binding activity of the recombinant antibody in the culture supernatant are measured using an enzyme immunoassay antibody method [Monoclonal Antibodies-Principles and practice, Third edition, Academic Press (1996); Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988), monoclonal antibody laboratory manual, Kodansha Scientific (1987)] or the like.

(8) Acquisition of transformant which stably expresses recombinant antibody and preparation of recombinant antibody

[0234] A transformant which stably expresses a recombinant antibody can be obtained by introducing the recombinant antibody expression vectors obtained in (3) and (6) described above into an appropriate host cell.

[0235] The expression vectors are introduced into a host cell using an electroporation method [JP-A-H02-257891; Cytotechnology, 3, 133(1990)] or the like.

[0236] The host cell into which a recombinant antibody expression vector is introduced is not particularly limited as long as the host cell is capable of expressing a recombinant antibody. For example, CHO-K1 (ATCC No: CCL-61), DUKXB11 (ATCC No: CCL-9096), Pro-5 (ATCC No: CCL-1781), CHO-S (Life Technologies, Cat #11619), rat myeloma cells YB2/3HL. P2. G11. 16Ag. 20 (also referred to as YB2/0), mouse myeloma cells NS0, mouse myeloma cells SP2/0-Ag14 (ATCC No: CRL1581), mouse P3-X63-Ag8653 cells (ATCC No: CRL1580), CHO cells [Proc. Natl. Acad. Sci. USA, 77, 4216 (1980)] in which dihydrofolate reductase (hereinafter, referred to as DHFR) genes are deficient, Lec13 obtaining lectin-resistance [Somatic Cell and Molecular Genetics, 12, 55 (1986)], CHO cells in which α 1,6-fucose transferase genes are deficient (Pamphlet of International Publication No.2005/035586 and Pamphlet of International Publication No.02/31140), or rat YB2/3HL. P2. G11. 16Ag. 20 cells (ATCC No: CRL1662) are used.

[0237] After introduction of an expression vector, the transformant which stably expresses a recombinant antibody is selected by culturing in a culture medium for culturing animal cells including an agent such as G418 sulfate (JP-A-

H02-257891)).

[0238] As the culture medium for culturing animal cells, RPMI1640 culture medium (Invitrogen), GIT culture medium (Nihon Pharmaceutical Co., Ltd.), EX-CELL301 culture medium (Jay Earl H., Inc.), IMDM culture medium (Invitrogen), Hybridoma-SFM culture medium (Invitrogen), or culture media obtained by adding various additives such as FBS to these culture media is used.

[0239] A recombinant antibody is produced and accumulated in the culture supernatant by culturing an obtained transformant in a culture medium. The expression amount and the antigen-binding activity of a recombinant antibody in the culture supernatant can be measured by an ELISA method. Further, the expression amount of a recombinant antibody produced by a transformant can be increased using a DHFR amplification system (JP-A-H02-257891) or the like.

[0240] The recombinant antibody is purified using a protein A-column from the culture supernatant of a transformant [Monoclonal Antibodies-Principles and Practice, Third edition, Academic Press (1996); Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)]. Moreover, methods used for protein purification such as gel filtration, ion exchange chromatography, and ultrafiltration may be used in combination.

[0241] The molecular weight of the H chain, the L chain, or the entire antibody molecules of the purified recombinant antibody can be measured using a polyacrylamide gel electrophoresis method [Nature, 227, 680 (1970)], a western blotting method [Monoclonal Antibodies-Principles and Practice, Third edition, Academic Press (1996); Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)] or the like.

3. Activity evaluation of purified monoclonal antibody or antibody fragment thereof

[0242] The activity of the purified monoclonal antibody or the antibody fragment thereof of the present invention can be evaluated in the following manner.

[0243] The binding activity against the human CRTH2-expressing cell line can be measured using a fluorescent antibody method [Cancer Immunol. Immunother., 36, 373 (1993)] such as a binding reaction-detecting system using the flow cytometry in 1. (6) described above.

[0244] The CDC activity or the ADCC activity against the human CRTH2-positive cultured cell line is measured by a known measurement method [Cancer Immunol. Immunother., 36, 373 (1993); Current protocols in Immunology, Chapter 7. Immunologic studies in humans, Editor, John E. Coligan et al., John Wiley & Sons, Inc., (1993)].

4. Method of controlling effector activity of antibody

[0245] As the method of controlling the effector activity of the monoclonal antibody of the present invention, a method of controlling the amount of fucose which is α 1,6-bound (also referred to as core fucose) to N-acetyl glucosamine (GlcNAc) present in the reducing terminal of a complex type N-linked sugar chain bound to 297th asparagine (Asn) in the Fc region of an antibody (Pamphlet of International Publication No.2005/035586, Pamphlet of International Publication No.2002/31140, and Pamphlet of International Publication No.00/61739) and a method of controlling the activity by modifying the amino acid residues in the Fc region of an antibody are known. The effector activity of the monoclonal antibody of the present invention can be controlled by any of the method.

[0246] The effector activity indicates antibody-dependent activity caused through the Fc region of an antibody, and ADCC activity, CDC activity, and antibody-dependent phagocytosis (ADP activity) by phagocytes such as macrophages or dendritic cells are known.

[0247] As an example of the method of measuring effector activity, inflammatory cells as a target, human peripheral blood mononuclear cells (PBMC) as an effector, and inflammatory cell-specific antibody are mixed with each other and incubated for approximately 4 hours, and then lactate dehydrogenase (LDH) released as an index of cell injury can be measured. Alternatively, an antibody that recognizes a blood cell-specific antigen such as CD20 is mixed with human PBMC and incubated, then released LDH and a decrease in number of cells as effector activity can be measured using flow cytometry.

[0248] The effector activity of an antibody can increase or decrease by controlling the content of core fucose in the complex type N-linked sugar chain of Fc of the antibody. A method of expressing an antibody using CHO cells in which α 1,6-fucose transferase genes are deficient may be exemplified as the method of decreasing the content of fucose bound to the complex type N-linked sugar chain which binds to Fc of the antibody, and an antibody to which fucose does not bind can be obtained. The antibody to which fucose does not bind has high ADCC activity.

[0249] On the other hand, method of expressing an antibody using a host cell into which α 1,6-fucose transferase genes are introduced may be exemplified as the method of increasing the content of fucose bound to the complex type N-linked sugar chain which binds to Fc of the antibody, and an antibody to which fucose binds can be obtained. The antibody to which fucose binds has lower ADCC activity than the antibody to which fucose does not bind.

[0250] Moreover, the ADCC activity or the CDC activity can increase or decrease by modifying the amino acid residues in the Fc region of an antibody. For example, the CDC activity of an antibody can be increased using the amino acid

sequences in the Fc region described in US-A-2007/0148165.

[0251] In addition, the ADCC activity or the CDC activity can be increased or decreased by performing amino acid modification described in US Patent No. 6737056, US Patent No. 7297775, or US Patent No. 7317091.

[0252] The antibodies of the present invention include an antibody whose half-life in blood is controlled by performing amino acid modification described in JP-A-2013-165716 or JP-A-2012-021004 or the like according to the amino acid modification or sugar chain modification in the antibody constant region described above to control the reactivity to an Fc receptor.

[0253] Further, an antibody whose effector activity or half-life in blood is controlled can be obtained by combining the above-described methods and using the combined method for one antibody.

5. Method of treating disease using monoclonal antibody or antibody fragment thereof of the present invention

[0254] The antibody of the present invention may be used for treating a disease associated with human CRTH2. Examples of administration routes include oral administration and parenteral administration such as oral cavity administration, tracheobronchial administration, rectally administration, subcutaneous administration, intramuscular administration, intravenous administration, or intraperitoneal administration. Examples of administration forms include sprays, capsules, tablets, powders, granules, syrups, emulsions, suppositories, injections, ointments, and tapes.

[0255] Examples of the formulation suitable for oral administration include emulsions, syrups, capsules, tablets, powders, and granules.

[0256] Liquid preparations such as emulsions or syrups are produced using, as additives, water; saccharides such as sucrose, sorbitol, and fructose; glycols such as polyethylene glycol and propylene glycol; oils such as sesame oil, olive oil, and soybean oil; preservatives such as p-hydroxybenzoic acid esters; and flavors such as strawberry flavor and peppermint.

[0257] Capsules, tablets, powders, or granules are produced using, as additives, excipients such as lactose, glucose, sucrose, and mannitol; disintegrating agent such as starch and sodium alginate; lubricants such as magnesium stearate and talc; binders such as polyvinyl alcohol, hydroxy propyl cellulose, and gelatin; surfactants such as fatty acid ester; and plasticizers such as glycerin.

[0258] Examples of the formulation suitable for parenteral administration include injections, suppositories, and sprays.

[0259] An injection is produced using a carrier formed of a salt solution, a glucose solution, or a mixture of these.

[0260] A suppository is produced using a carrier such as cacao butter, hydrogenated fats, or carboxylic acid.

[0261] A spray is produced using a carrier which doesn't stimulate the oral cavity and the respiratory tract mucosa of a recipient, and enables the monoclonal antibody or the antibody fragment thereof of the present invention to disperse as fine particles to be easily absorbed. As the carrier, for example, lactose or glycerin is used. Further, a spray can be produced as an aerosol or dry powder.

[0262] Moreover, the components exemplified as additives for oral administration can be also added to the above-described parenteral agents.

6. Method of diagnosing disease using antibody or antibody fragment thereof of the present invention

[0263] A disease associated with human CRTH2 can be diagnosed by detecting or measuring human CRTH2 or human CRTH2-expressing cells using the antibody or the antibody fragment thereof of the present invention.

[0264] The allergic disease which is one of the diseases associated with human CRTH2 can be diagnosed, for example, by detecting human CRTH2 expressed on inflammatory cells present in peripheral blood, sputum, nasal discharge, or bronchoalveolar lavage derived from patients using an immunological technique such as flow cytometer.

[0265] The immunological technique is a method of detecting or measuring the amount of antibody or the amount of antigen using a labeled antigen or antibody. For example, a radioactive substance-labeled immunoantibody method, an enzyme immunoassay method, a fluorescent immunoassay method, a luminescent immunoassay method, a western blotting method, and a physico-chemical method are used.

[0266] The radioactive substance-labeled immunoantibody method is carried out, for example, by reacting the antibody or the antibody fragment thereof of the present invention with an antigen or cells expressing an antigen, reacting a radiation-labeled anti-immunoglobulin antibody or a binding fragment with the antigen or the cells, and then measurement is carried out using a scintillation counter or the like.

[0267] Examples of the enzyme immunoassay method include a method in which the antibody or the antibody fragment thereof of the present invention is allowed to react with an antigen or cells expressing an antigen or the like, then an anti-immunoglobulin antibody or a binding fragment which has been labeled thereof is allowed to react therewith, and then the coloring dye is measured using a spectrophotometer. For example, a sandwich ELISA method or the like is used.

[0268] As a marker used for the enzyme immunoassay method, known enzyme labeling [Enzyme Immunoassay method, Igaku-Shoin Ltd. (1987)] can be used. For example, alkaline phosphatase labeling, peroxidase labeling, luci-

ferase labeling, or biotin labeling is used.

[0269] The sandwich ELISA method is a method of trapping an antigen which is a subject for detection or measurement after an antibody binds to a solid phase and reacting a second antibody with the trapped antigen. According to the ELISA method, two kinds of antibodies which are antibodies or the antibody fragments thereof recognizing an antibody intended to be detected or measured and have antigen recognition sites different from each other are prepared, the first antibody or the antibody fragment among these are adsorbed to a plate (for example, 96-well plate) in advance, and then the second antibody or the antibody fragment is labeled using a fluorescent substance such as FITC, an enzyme such as peroxidase, biotin, or the like.

[0270] Cells or a disrupted liquid thereof, tissues or a disrupted liquid thereof, a cell culture supernatant, serum, pleural effusion, ascites fluid, or intraocular fluid, which are separated from a living body are allowed to react with the plate to which the above-described antibody is adsorbed, then allowed to react the labeled monoclonal antibody or the antibody fragment thereof with the plate, and then a detection reaction is carried out corresponding to the labeling substance. The antigen concentration in a test sample is calculated using a calibration curve prepared by gradually diluting the antigen of a known concentration.

[0271] As the antibody used for the sandwich ELISA method, any of a polyclonal antibody or a monoclonal antibody may be used, and an antibody fragment such as Fab, Fab', or F(ab')₂ may be used. As the combination of two kinds of antibodies used for the sandwich ELISA method, a combination of monoclonal antibodies or antibody fragments thereof recognizing different epitopes may be used or a combination of a polyclonal antibody and a monoclonal antibody or an antibody fragment thereof may be used.

[0272] A fluorescence immunoassay method is carried out by a method described in literature [Monoclonal Antibodies-Principles and practice, Third edition, Academic Press (1996), monoclonal antibody experiment manual, Kodansha Scientific (1987)] or the like. As the label used for the fluorescence immunoassay method include known fluorescent labels [fluorescent antibody method, Soft Science Inc. (1983)] may be exemplified. Further, fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (RITC) may be exemplified.

[0273] A luminescent immunoassay method is carried out by a method described in literature [bioluminescence and chemiluminescence clinical examination 42, Hirokawa Shoten (1998)] or the like. As a marker used for the luminescent immunoassay method, known luminescent label may be exemplified. Examples thereof include acridinium ester and lophine.

[0274] Western blotting method is a method in which an antigen or cells expressing an antigen is fractionated by sodium dodecyl sulfate (SDS)-PAGE [Antibodies-A Laboratory Manual Cold Spring Harbor Laboratory (1988)], the gel is blotted onto a polyvinylidene fluoride (PVDF) membrane or a nitrocellulose membrane, the membrane is allowed to react with an antigen-recognizing antibody or the antibody fragment, further allowed to react with an anti-mouse IgG antibody or a binding fragment which is labeled with a fluorescent substance such as FITC, enzyme such as peroxidase or biotin, or the like, and the label is visualized to confirm the reaction. An example will be described below.

[0275] Cells or tissues that express a polypeptide comprising the amino acid sequence represented by SEQ ID NO: 2 are dissolved and 0.1 to 30 µg of protein per lane is electrophoresed under reducing conditions by an SDS-PAGE method. A blocking operation is performed by transferring the electrophoresed protein to a PVDF membrane and causing a reaction with 1% to 10% BSA-PBS at room temperature for 30 minutes.

[0276] Here, the antibody of the present invention is reacted and washed with PBS containing 0.05% to 0.1% of Tween-20 (hereinafter, referred to as Tween-PBS), and then a peroxidase-labeled goat anti-mouse IgG is reacted at room temperature for 2 hours. It is washed with Tween-PBS and a band to which a monoclonal antibody binds is detected using ECL Western Blotting Detection Reagents (manufactured by Amersham plc) or the like to detect a polypeptide comprising the amino acid sequence represented by SEQ ID NO: 2. As the antibody used for detection in western blotting, an antibody which can bind to a polypeptide that does not have a natural type three-dimensional structure is used.

[0277] The physico-chemical method is performed by binding the monoclonal antibody or the antibody fragment thereof of the present invention to human CRTH2 which is an antigen to form an aggregate and detecting the aggregate. Other examples of the physico-chemical method include a capillary tube method, a one-dimensional immunodiffusion method, an immunoturbidimetry method, and a latex immunoturbidimetry method [Kanai's Manual of Clinical Laboratory Medicine, Kanehara-Shuppan (1998)].

[0278] In a latex immunoturbidimetry method, when an antigen-antibody reaction is carried out by the corresponding antigen or antibody using a carrier such as polystyrene latex in which an antibody or an antigen is sensitized and which has a particle diameter of approximately 0.1 to 1 µm, scattered light in the reaction solution increases and transmitted light decreases. The antigen concentration in a test sample or the like is measured by detecting this change as the absorbance or integrate sphere turbidity.

[0279] The method of determining therapeutic efficacy, of the antibody before treatment is started, using the antibody or the antibody fragment thereof of the present invention is exemplified as follows.

[0280] First, before treatment is started, the peripheral blood, sputum, bronchoalveolar lavage, and nasal discharge or the like are collected from a patient, the antibody or the antibody fragment thereof of the present invention is added

to the suspension, and anti-inflammatory cell activity including removal of inflammatory cells and inhibitory activity against biofunctional molecules such as Th2 type cytokine is measured after a certain period of time. In a case where anti-inflammatory cell activity is detected as the result of measurement, it is possible to determine, before treatment, that the antibody or the antibody fragment thereof of the present invention is effective for the treatment for patients having the peripheral blood, sputum, nasal discharge or the like.

Examples

[0281] Hereinafter, the present invention is described by examples, but the present invention is not limited thereto.

[Example 1]

Establishment of human CRTH2-expressing cells

(1) Preparation of human CRTH2 expression vector

[0282] cDNA of human CRTH2 (hereinafter, also referred to as CRTH2) was entirely synthesized and used for the following test. The cDNA sequence of human CRTH2 is represented by SEQ ID NO: 1 and the amino acid sequence thereof is represented by SEQ ID NO: 2.

(i) Construction of human CRTH2 gene expression pKANTEX93 vector

[0283] A human CRTH2 gene expression pKANTEX93 vector was constructed by connecting cDNA of human CRTH2 with a vector pKANTEX93 (Pamphlet of International Publication No.97/10354) using restriction enzymes EcoRI and KpnI.

(ii) Construction of human CRTH2 gene expression pAMoh vector

[0284] A human CRTH2 gene expression pAMoh vector was constructed by incorporating human CRTH2 genes in pAMoh (Pamphlet of International Publication No.03/087366) using restriction enzymes KpnI and HindIII.

(2) Establishment of human CRTH2-expressing CHO/DG44 cells

[0285] DHFR gene-deficient CHO (Chinese hamster ovary) cell DG44 lines (CHO/DG44 cells) were obtained from Mitsubishi Chemical Corporation, Yokohama Research Institute. and used for establishment of human CRTH2-expressing cells. IMDM (GIBCO Inc.) to which 10% dialyzed fetal bovine serum (dFBS) (GIBCO Inc.), HT [hypoxanthine (H) and thymidine (T)] supplement (GIBCO Inc.) and 50 μ g/mL of gentamicin (NACALAI TESQUE, Inc.) was added (hereinafter, abbreviated as IMDM culture medium) were used for culturing.

[0286] First, the human CRTH2 gene expression pKANTEX93 vector prepared in (1)-(i) was cleaved by restriction enzyme AatII treatment and then the obtained linear DNA was purified and dissolved in sterile water. This DNA was introduced to CHO/DG44 cells by an electroporation method and cultured in IMDM culture medium from which HT supplement was removed for approximately 3 days.

[0287] Thereafter, drug-resistant cells were selected using IMDM to which 10% dFBS, 0.5 mg/mL of G418 (NACALAI TESQUE, Inc.), and 50 μ g/mL of gentamicin (NACALAI TESQUE, Inc.) were added (hereinafter, abbreviated as IMDM selection medium). The selected drug-resistant cells were seeded in a 96-well plate at 75 cells/plate and cultured in IMDM selection medium for approximately 2 weeks. The cells in each well were observed using a microscope and a single clone was sequentially expanded.

[0288] The obtained drug-resistant cells were peeled using a 0.02% EDTA solution (NACALAI TESQUE, Inc.), washed with phosphate buffered saline (PBS), and suspended with PBS containing 2% fetal bovine serum (FBS), 0.05% NaN₃, and 1 mM EDTA (staining medium, hereinafter, abbreviated as SM). Next, the cells were seeded in a 96-well plate such that each well had 2×10^5 cells and centrifuged at 1700 rpm for 2 minutes.

[0289] After the supernatant was removed, a PE-labeled anti-human CRTH2 antibody (Beckman Coulter Inc.) prepared using SM was added thereto to cause a reaction at 4°C for 1 hour. The cells were washed and suspended with SM, and the fluorescent intensity was analyzed by flow cytometer (BD Biosciences, FACS Cantoll). A clone which expressed human CRTH2 at a high level was selected and this cell was set to human CRTH2 expressing CHO/DG44 cell.

(3) Preparation of FLAG fusion human CRTH2 expression vector

(i) Construction of FLAG fusion human CRTH2 gene expression pAMoh vector

[0290] Human CRTH2 (SEQ ID NO: 5) with a C terminal to which a FLAG tag was added was amplified by polymerase chain reaction (hereinafter, referred to as PCR) from the human CRTH2 gene expression pAMoh prepared in (1)-(ii) using primer human CRTH2FLAG-A (SEQ ID NO: 3) and human CRTH2 FLAG-B (SEQ ID NO: 4) and was connected to a vector pAMOH (Pamphlet of International Publication No.03/087366) using restriction enzymes KpnI and HindIII, thereby constructing a FLAG fusion human CRTH2 gene expression pAMoh vector.

(ii) Construction of FLAG fusion human CRTH2 expression vector pKANTEX93 vector

[0291] Human CRTH2 with a C terminal to which a FLAG tag was added was amplified by PCR from the FLAG fusion human CRTH2 gene expression pAMoh vector prepared in (3)-(i) using primer human CRTH2FLAG-C (SEQ ID NO: 6) and human CRTH2 FLAG-D (SEQ ID NO: 7) and was connected to a vector pKANTEX93 (Pamphlet of International Publication No.97/10354) using restriction enzymes EcoRI and KpnI, thereby constructing a FLAG fusion human CRTH2 gene expression pKANTEX93 vector.

(4) Establishment of FLAG fusion human CRTH2 expressing 3Y1-B cells

[0292] Rat 3Y1-B cells were obtained from Riken BioResource Center and used for establishment of FLAG fusion human CRTH2-expressing cells. 10% FBS (GIBCO Inc.) and DMEM (GIBCO Inc.) to which 50 µg/mL of gentamicin (NACALAI TESQUE, Inc.) was added (hereinafter, abbreviated as DMEM culture medium) were used for culturing.

[0293] The FLAG fusion human CRTH2 gene expression pKANTEX93 vector prepared in (3)-(ii) was cleaved by restriction enzyme AatII treatment and then the obtained linear DNA was purified and dissolved in sterile water. This DNA was introduced to 3Y1-B cells according to a Lipofection method using Eugene6 (Promega Corporation) and cultured in DMEM culture medium for 3 days. Thereafter, drug-resistant cells were selected using the DMEM to which 10% FBS, 0.8 mg/mL of G418 (NACALAI TESQUE, Inc.), and 50 µg/mL of gentamicin (NACALAI TESQUE, Inc.) were added (hereinafter, abbreviated as DMEM selection medium).

[0294] The obtained drug-resistant cells were peeled using a 0.05% trypsin solution (Invitrogen), washed with PBS, and suspended with SM. Next, the cells were seeded in a 96-well plate at 2×10^5 cells/well and centrifuged at 1700 rpm for 2 minutes. After the supernatant was removed, a PE-labeled anti-human CRTH2 antibody (Beckman Coulter Inc.) prepared using SM was added thereto to cause a reaction at 4°C for 1 hour. The cells were washed and suspended by SM, and the fluorescent intensity was analyzed by flow cytometer (BD Biosciences, FACS Aria). A fraction which expressed FLAG fusion human CRTH2 at a high level was sorted out and expansively cultured, and this cell was set to FLAG fusion human CRTH2 expressing 3Y1-B cell.

[Example 2]

Preparation of antibody monoclonal antibody against human CRTH2

(1) Immunization to rat

[0295] In order to obtain a monoclonal antibody against human CRTH2, a female WKY/NCrCrlj rat (WKY rat) (Charles River Laboratories) who was 9 weeks old at the time of first administration was immunized.

[0296] At the time of first administration, 5×10^6 cells of FLAG fusion human CRTH2-expressing 3Y1-B cells were suspended in 100 µL of physiological saline (Otsuka Pharmaceutical factory Inc.) and combined with 100 µL of Sigma Adjuvant System (registered trademark) (Sigma-Aldrich Co. LLC) to prepare 200 µL of cell suspension, and then 100 µL of the cell suspension was administered intramuscularly to both right and left portions of the base of the tail of a WKY rat.

[0297] After 2 weeks from the first administration, 5×10^6 cells of FLAG fusion human CRTH2-expressing 3Y1-B cells were suspended in a 200 µL physiological saline and administered in the same manner as described above.

(2) Preparation of hybridomas

[0298] After 3 days from the second immunization described in (1), iliac lymph nodes were surgically removed from the WKY rat for cell fusion.

[0299] First, the removed iliac lymph nodes were ground using slide glass so that the tissues were loosened. The iliac lymph node tissues were suspended by Minimum Essential Media (MEM) (Invitrogen) and unnecessary tissues were

removed by passing through a cell strainer. The supernatant was removed by centrifugation at 1200 rpm for 5 minutes and resuspended with MEM to obtain iliac lymph node cells.

[0300] To the obtained iliac lymph node cells, 1/5 number of mouse myeloma cell lines P3-U1 (ATCC) were added. The supernatant was removed by centrifugation and warmed in a warm bath at 37°C, 500 µL of a PEG solution [solution obtained by mixing 1 mL of polyethylene glycol 1000 (Junsei Chemical Co., Ltd.) with 1 mL of MEM and adding 350 µL of DMSO (dimethyl sulfoxide) (Sigma-Aldrich Co. LLC) thereto] was gently added thereto, and 1 mL of MEM was added every minute for five times and then 45 mL of MEM was added thereto.

[0301] The supernatant was removed by centrifugation at 900 rpm for 5 minutes, and the cells were suspended with HAT culture medium [500 mL of RPMI-1640 (Wako Pure Chemical Industries) to which 10 mL of HAT (hypoxanthine (H), aminopterin (A), and thymidine (T)) solution (GIBCO Inc.), 0.5 mL of 55 mmol/L 2-mercaptoethanol (Invitrogen), 50 mL of fetal bovine serum (Moregate Biotech), and 0.5 mL of 10 mg/mL gentamicin solution (NACALAI TESQUE, Inc.) were added], seeded in a 96-well plate, and cultured.

(3) Hybridoma screening

[0302] After hybridomas seeded in (2) were cultured for 7 days, the culture supernatant of each well was collected and the reactivity against human CRTH2 was analyzed. Human CRTH2 expressing CHO/DG44 cells and CHO/DG44 cells were respectively set to the positive control cells and the negative control cells. First, the positive control cells and the negative control cells were peeled using 0.02% EDTA solution (NACALAI TESQUE, Inc.) and seeded in a 96-well plate at 1×10^5 cells/50 µL for each well, and 50 µL of the culture supernatant was added thereto to cause a reaction at 4°C for 30 minutes.

[0303] The cells were washed and suspended with 100 µL of Anti-rat IgG (Fc)-Dylight488 (Abeam plc.) diluted 300 times with SM to cause a reaction at 4°C for 30 minutes. The cells were washed again and suspended with SM, and then the fluorescent intensity was analyzed by flow cytometry (BD Biosciences, FACS Cantoll).

[0304] Single cell cloning was performed twice on hybridomas in wells specifically reacting with human CRTH2 expressing CHO/DG44 cells using a cloning culture medium [S Medium Cloning Medium CM-B (EIDIA Co., Ltd.) to which 0.5 mL of 10 mg/mL gentamicin (NACALAI TESQUE, Inc.) and 5 mL of HT supplement (GIBCO Inc.) was added] by a limiting dilution method. Finally, hybridoma Lym2 clone (hereinafter, referred to as hybridoma Lym2) showing strongest flow cytometry reactivity against human CRTH2 expressing CHO/DG44 cells was established.

(4) Identification of subclass of antibody contained in culture supernatant of hybridoma Lym2

[0305] The culture supernatant obtained by culturing hybridoma Lym2 for 3 days was diluted 10 times with PBS (NACALAI TESQUE, Inc.) and the subclass was analyzed by Rat Monoclonal Antibody Isotyping Test Kit (Abd Serotec Inc.) according to the attached instructions using 150 µL of the diluent.

[0306] As the result, the rat anti-human CRTH2 monoclonal antibody contained in the culture supernatant of hybridoma Lym2 (hereinafter, also simply abbreviated as Lym2 antibody) was found to be a rat IgG2b antibody.

(5) Purification of Lym2 antibody

[0307] The hybridoma Lym2 was cultured for 1 week using a culture medium obtained by adding 5% Fetal Bovine Serum Ultra Low IgG (Invitrogen) to Hybridoma-SFM (Invitrogen). The culture supernatant was recovered for purification.

[0308] The Lym2 antibody was purified from the culture supernatant using Prosep-G (GE Healthcare). First, the culture supernatant was loaded onto a column, and the column was washed with PBS and eluted by elution buffers having pHs of 5.0, 3.5, and 3.0 (0.1 M citric acid monohydrate-NaOH/pH 5.0, 3.5, and 3.0) in this order. The eluted fractions were immediately neutralized by a neutralization buffer (2 M Tris-HCl/pH 8.5).

[0309] The absorbance (280 nm) of each fraction was measured and a series of fractions having high measurement values were recovered as an antibody fraction. The antibody fraction was dialyzed with PBS and allowed to pass through a filter, 0.22 µm to obtain purified protein. The absorption coefficient at 280 nm was set to 1.4 and the concentration was calculated.

[Example 3]

Evaluation of antigen-binding properties of Lym2 antibody using flow cytometry

[0310] FLAG fusion human CRTH2-expressing 3Y1-B cells were peeled using a 0.05% trypsin solution (Invitrogen), washed with PBS, and suspended with SM. Further, the cells were seeded in a 96-well plate such that one well has 2×10^5 cells and centrifuged at 1700 rpm for 2 minutes. After the supernatant was removed, 100 µL of Lym2 antibody

prepared to have a concentration of 10 µg/mL using SM was added thereto to cause a reaction at 4°C for 1 hour.

[0311] After the cells were washed, Anti-rat IgG-FITC (Beckman Coulter Inc.) diluted 100 times with SM was added to the cells to cause a reaction at 4°C for 1 hour. The cells were washed again and suspended with SM, and the fluorescent intensity was analyzed by flow cytometry (BD Biosciences, FACS Cantoll). As the result, binding of the Lym2 antibody to FLAG fusion human CRTH2-expressing 3Y1-B cells was observed.

[Example 4]

Cloning of gene of heavy chain and light chain variable region of Lym2 antibody

[0312] The hybridoma Lym2 washed with PBS was dissolved using RNAiso plus (TAKARA BIO INC.) according to attached instructions, thereby preparing total RNA. The obtained total RNA was dissolved in DEPC treated water (Invitrogen). Next, mRNA was purified from the obtained total RNA using Oligotex-dT30 <Super>mRNA Purification Kit (Fromtotal RNA) (TAKARA BIO INC.) according to attached instructions. Then, cDNA was prepared from the purified mRNA using SMART RACE cDNA Amplification Kit (Clontech Laboratories, Inc.) according to attached instructions.

[0313] Using obtained cDNA as a template, genes of rat IgG2b heavy chain were amplified by PCR using primer Rat IgG2bH-A (SEQ ID NO: 8) and Rat IgG2bH-B (SEQ ID NO: 9) and genes of rat light chain (κ chain) were amplified by PCR using primer Ratk-A (SEQ ID NO: 10) and Ratk-B (SEQ ID NO: 11). Sub-cloning was performed on the amplified genes and the base sequences were analyzed.

[0314] As the result, the base sequences and amino acid sequences of VH and VL of Lym2 antibody including signal sequences were identified. The base sequences of VH and VL were represented by SEQ ID NOS: 12 and 14 respectively and the amino acid sequences thereof were represented by SEQ ID NOS: 13 and 15 respectively. Moreover, the sequences of VH and VL of Lym2 antibody which did not have signal sequences were identified based on the report of Kabat et al. [Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)].

[0315] The base sequences of VH and VL from which signal sequences were removed were represented by SEQ ID NOS: 16 and 18 respectively and the amino acid sequences were represented by SEQ ID NOS: 17 and 19 respectively. The amino acid sequences of CDR1, CDR2, and CDR3 of VH of Lym2 antibody were represented by SEQ ID NOS: 20, 21, and 22 respectively and the amino acid sequences of CDR1, CDR2, and CDR3 of VL of Lym2 antibody were represented by SEQ ID NOS: 23, 24, and 25 respectively.

[Example 5]

Preparation of rat/human chimeric Lym2 antibody

(1) Construction of rat/human chimeric Lym2 antibody expression vector

[0316] A rat/human chimeric Lym2 antibody expression vector was prepared by respectively connecting gene of heavy chain and a light chain variable region of Lym2 antibody to gene of heavy chain and a κ chain constant region of IgG1 using the following method.

[0317] First, the sequence of SEQ ID NO: 26 as VH and the sequence of SEQ ID NO: 27 as VL were entirely synthesized. From the synthesized sequences, the VH was amplified by PCR using a primer chLym2VH-A (SEQ ID NO: 28) and a primer chLym2VH-B (SEQ ID NO: 29) and the VL was amplified by PCR using a primer chLym2VL-A (SEQ ID NO: 30) and a primer chLym2VL-B (SEQ ID NO: 31).

[0318] A gene fragment was subjected to agarose gel electrophoresis and purified by QIAquick Gel Extraction Kit (QIAGEN). Sub-cloning to vectors by In-Fusion HD Cloning Kit (Clontech Laboratories, Inc.) was performed using this fragment, a human κ constant region expression vector (Bg1II/BsiWI treatment), and a human heavy chain (IgG1) constant region expression vector (Sall/NheI treatment) according to the attached instructions.

[0319] A rat/human chimeric Lym2 antibody (hereinafter, referred to as chLym2) expression vector was prepared by performing transformation to E. coli DH5α competent cells (TAKARA BIO INC.), plasmid extraction, and sequence confirmation.

(2) Preparation of chLym2 transiently expressing cell line

[0320] In order to prepare chLym2 transiently expressing cell line, the expression vector prepared in (1) was introduced into a host cell according to the following method using Freestyle (registered trademark) MAX CHO Expression System (Lifetechnologies Corporation) according to the attached instructions. FUT8 knockout CHO cells (Pamphlet of International Publication No.2005/035586 and Pamphlet of International Publication No.02/31140) which were acclimated to FreeStyle (registered trademark) CHO Expression Medium (Lifetechnologies Corporation) were used as the host cell.

[0321] 312.5 μ g of chLym2 antibody expression vector (vector obtained by mixing a light chain expression vector with a heavy chain expression vector at a mixing ratio of 1:2) prepared in (1) was dissolved in 20 mL of Opti-Pro SFM (Invitrogen) and 312.5 μ L of Freestyle MAX Reagent (Invitrogen) was dissolved in 20 mL of Opti-Pro SFM and these two liquids were allowed to stand at room temperature for 5 minutes. The two liquids were mixed with each other and allowed to stand at room temperature for 15 minutes. All of the mixed solution was added to 250 mL of host cell culture solution (1×10^6 cells/mL), thereby obtaining a chLym2 transiently expressing cell line.

(3) Purification of chLym2

[0322] ChLym2 transiently expressing cell line obtained in (2) was suspended with Free style CHO expression medium (Invitrogen) to which 8 mM of L-glutamine (Invitrogen) was added and cultured using an Erlenmeyer flask for 5 days, and the culture supernatant was recovered. The recovered culture supernatant was centrifuged and filtered using a filter, 0.22 μ m, thereby preparing a culture supernatant containing chLym2.

[0323] ChLym2 was purified from the prepared culture supernatant using MabSelect SuRe (GE Healthcare). First, the culture supernatant was loaded onto a column, and the column was washed with PBS and eluted by elution buffers having pHs of 5.0, 3.5, and 3.0 (0.1 M citric acid monohydrate-NaOH/pH 5.0, 3.5, and 3.0) in this order. The eluted fractions were immediately neutralized with a neutralization buffer (2 M Tris-HCl/pH 8.5).

[0324] The absorbance at 280 nm (A_{280}) of each fraction was measured and a series of fractions having high measurement values were recovered as an antibody fraction. The antibody fraction was dialyzed with PBS and allowed to pass through a filter, 0.22 μ m to obtain purified protein. The absorption coefficient at 280 nm was set to 1.37 and the concentration was calculated.

[Example 6]

Preparation of humanized antibody

(1) Design of heavy chain and light chain variable region of humanized Lym2 antibody

(i) Design of amino acid sequences of VL and VH of humanized Lym2 antibody

[0325] The amino acid sequences of VL of humanized Lym2 antibodies were designed in the following manner.

[0326] First, an amino acid sequence of FR of VL of a human antibody suitable for transplantation of the amino acid sequences of CDRs 1 to 3 of VL of the Lym2 antibody (SEQ ID NOS: 23, 24, and 25) was selected in the following manner.

[0327] In a case of known human antibody heavy chain variable region sequences, a human antibody sequence having high homology with the FR sequence of VL of Lym2 antibody was searched using BLASTP database provided by The National Center for Biotechnology Information. As the result, the human antibody sequence of GeneBank ID: ABA71374.1 exhibited the highest homology, so the FR of this antibody was selected. LV0 (SEQ ID NO: 33) was designed by transplanting the amino acid sequences of CDRs 1 to 3 of VL of Lym2 antibody represented by SEQ ID NOS: 23, 24, and 25 into suitable positions in the human antibody FR sequences determined in the above-described manner.

[0328] Next, the amino acid sequences of VH of the humanized Lym2 antibody were designed in the following manner. An amino acid sequence of FR of VH of a human antibody suitable for transplantation of the amino acid sequences of CDRs 1 to 3 of VH of the Lym2 antibody (SEQ ID NOS: 20, 21, and 22) was selected in the following manner. Similar to VL, a human antibody sequence having high homology with the FR sequence of VH of Lym2 antibody was searched based on the BLASTP database.

[0329] As the result, the human antibody sequence of GeneBank ID: AAY33331.1 exhibited the highest homology, so the FR of this antibody was selected. HV0 (SEQ ID NO: 49) was designed by transplanting the amino acid sequences of CDRs 1 to 3 of VH of Lym2 antibody represented by SEQ ID NOS: 20, 21, and 22 into suitable positions in the human antibody FR sequences determined in the above-described manner.

[0330] LV0 which is the amino acid sequence of VL of the humanized Lym2 antibody and HV0 which is the amino acid sequence of VH of the humanized Lym2 antibody designed in the above-described manner are sequences obtained by transplanting only the amino acid sequences of CDRs derived from Lym2 which is a rat monoclonal antibody to the amino acid sequence of FR of the selected human antibody. However, typically, in a case of preparing a humanized antibody, the binding activity frequently decreases when the amino acid sequences of CDRs of a rodent-derived antibody are simply transplanted into the FR of a human antibody.

[0331] In order to avoid the decrease of binding activity, amino acid residues which are considered to affect the binding activity, among the amino acid residues of FR which are different between the human antibody and the rodent-derived antibody, are substituted along with the transplantation of the amino acid sequences of CDRs. In the present example, amino acid residues of FR which are considered to affect the binding activity were identified and substituted in the

following manner.

[0332] First, a three-dimensional structure of an antibody variable region formed by LV0 which is the amino acid sequence of VL of the humanized Lym2 antibody and HV0 which is the amino acid sequence of VH of the humanized Lym2 antibody designed in the above-described manner (hereinafter, referred to as LV0HV0) was constructed using a computer modeling technique.

[0333] Discovery Studio (Accelrys) was used for preparing a three-dimensional structure coordinate and displaying a three-dimensional structure. In addition, a computer model of a three-dimensional structure of the variable region of Lym2 antibody was constructed in the same manner. Further, amino acid residues which were different from those of Lym2 antibody were selected from the amino acid sequences of FR of VL and VH of LV0HV0, amino acid sequences modified to amino acid residues of the Lym2 antibody were prepared, and then a three-dimensional structure model was constructed in the same manner as described above. Amino acid residues which are expected to affect the binding activity of the antibodies were identified by comparing the prepared three-dimensional structures of each variable region of Lym2 antibody, LV0HV0, and variant.

[0334] As the result, as the amino acid residues among the amino acid residues of FR of LV0HV0 which are considered to change the three-dimensional structures of antigen-binding sites and affect the binding activity of the antibodies, 2nd Ile, 4th Met, 15th Pro, and 85th Ala in the amino acid sequences of SEQ ID NO: 33 were selected in a case of LV0 and 18th Leu, 77th Asn, 93rd Val, and 117th Thr in the amino acid sequence of SEQ ID NO: 49 were selected in a case of HV0.

[0335] The VL and VH of a humanized antibody comprising various modifications were designed by performing amino acid modification, that is, substitution of at least one amino acid sequence among the selected amino acid residues with amino acid residues present in the same site of Lym2 antibody. Specifically, in a case of the VL, at least one modification among amino acid modifications of substituting 2nd Ile with Val, 4th Met with Leu, 15th Pro with Leu, or 85th Ala with Pro in the amino acid sequence of SEQ ID NO: 33 was introduced. In a case of the VH, at least one modification among amino acid modifications of substituting 18th Leu with Met, 77th Asn with Ser, 93rd Val with Thr, or 117th Thr with Val in the amino acid sequence of SEQ ID NO: 49 was introduced.

[0336] LV0HV0, LV1HV0, LV2aHV0, LV2bHV0, LV2cHV0, LV3aHV0, LV3bHV0, LV4HV0, LV0HV4, LV1HV4, LV2aHV4, LV2bHV4, LV2cHV4, LV3aHV4, LV3bHV4, LV4HV4, LV0HV1, LV0HV2a, LV0HV2b, and LV0HV3 were respectively designed as antibody variable regions of LV0HV0, or the humanized Lym2 antibody in which at least one amino acid residue present in FR of LV0HV0 is modified.

[0337] In the description below, humanized Lym2 antibodies comprising the above-described variable regions are respectively abbreviated as LV0HV0, LV1HV0, LV2aHV0, LV2bHV0, LV2cHV0, LV3aHV0, LV3bHV0, LV4HV0, LV0HV4, LV1HV4, LV2aHV4, LV2bHV4, LV2cHV4, LV3aHV4, LV3bHV4, LV4HV4, LV0HV1, LV0HV2a, LV0HV2b, and LV0HV3.

[0338] The amino acid sequences of the light chain variable region LV0 (SEQ ID NO: 33), LV1 (SEQ ID NO: 35), LV2a (SEQ ID NO: 37), LV2b (SEQ ID NO: 39), LV2c (SEQ ID NO: 41), LV3a (SEQ ID NO: 43), LV3b (SEQ ID NO: 45), LV4 (SEQ ID NO: 47), and the heavy chain variable region HV0 (SEQ ID NO: 49), HV1 (SEQ ID NO: 51), HV2a (SEQ ID NO: 53), HV2b (SEQ ID NO: 55), HV3 (SEQ ID NO: 57), and HV4 (SEQ ID NO: 59) are respectively shown in Figs. 1 and 2.

(ii) Design of gene of variable region of humanized Lym2 antibody

[0339] The base sequences which encode amino acid sequences of variable regions of a humanized antibody were designed using codons highly frequently used in animal cells. With these base sequences, construction of a humanized Lym2 antibody expression vector described below and expression of the corresponding antibody were carried out.

(2) Construction of humanized Lym2 antibody expression vector

[0340] Humanized Lym2 antibody expression vectors were constructed in the same manner as described in Example 5-(1). That is, DNA with the base sequences represented by SEQ ID NOS: 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, and 58, encoding the amino acid sequences represented by SEQ ID NOS: 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, and 59 is entirely synthesized and the corresponding VL and VH gene fragments were amplified by PCR using the primers represented by SEQ ID NOS: 28 to 31. The gene fragments were subjected to agarose gel electrophoresis and purified by QIAquick Gel Extraction Kit (QIAGEN).

[0341] The VL and VH fragments were respectively sub-cloned to a human κ constant region expression vector (BglII/BsiWI treatment) and a human heavy chain (IgG1) constant region expression vector (SalI/NheI treatment) using In-Fusion HD Cloning Kit (Clontech Laboratories, Inc.) according to the attached instructions. E. coli DH5 α competent cells (TAKARA BIO INC.) were transformed using the prepared vector, plasmid extraction and sequence confirmation were performed to select a colony into which a correct sequence was inserted, and then a large amount of plasmids were prepared for transient expression.

(3) Transient expression of humanized Lym2 antibody

[0342] Transient expression of the prepared humanized Lym2 antibody was performed using Freestyle (registered trademark) MAX CHO Expression System (Lifetechnologies Corporation) with CHO cells which are acclimated to Free-Style (registered trademark) CHO Expression Medium (Lifetechnologies Corporation) described in Example 5-(2) as a host cell. The method of plasmid introduction was performed according to the attached instructions. The light chain expression vector and the heavy chain expression vector were used after being mixed with each other at a mixing ratio of 1:2.

[0343] The amount of culture solution was 200 mL and the cells were cultured for 5 days under set condition of 37°C at 125 rpm in the presence of 8% CO₂. After culture, the cell suspension was centrifuged and allowed to pass through a filter, 0.2 µm (Thermo Scientific) to recover the culture supernatant including a humanized Lym2 antibody was recovered.

(4) Purification of humanized Lym2 antibody

[0344] The humanized Lym2 antibody was purified by affinity purification using MabSelect SuRe (GE Healthcare) as follows. After the resin was equilibrated with PBS, the culture supernatant obtained in (3) was loaded and washed twice with PBS.

[0345] After the washing, an antibody was eluted using an elution buffer (20 mM citric acid, 50 mM NaCl, pH 3.4) and one-tenth the total amount of a neutralization buffer (1 M phosphoric acid-NaOH, pH 7.0) was added thereto for neutralization. Next, buffer-substitution with PBS using NAP25 (GE Healthcare) was performed. The resultant was concentrated by ultrafiltration using Amicon Ultra-4 Centrifugal Filter Units (Millipore) and the absorbance at 280 nm (A₂₈₀) was measured using Nanodrop8000 (Thermo Scientific) for concentration measurement and preparation of the antibody solution.

[Example 7]

Antigen-binding activity of chLym2 and humanized Lym2 antibody

[0346] FLAG fusion human CRTH2-expressing 3Y1-B cells were peeled using a 0.25% trypsin-EDTA (NACALAI TESQUE, Inc.), washed with PBS, and suspended with SM. Next, the cells were seeded in a 96-well plate such that one well has 1×10^5 cells. Further, chLym2 or each of the humanized Lym2 antibodies was added at each final concentrations of 50000, 12500, 3125, 781, 195, 49, 12, and 3 ng/mL to cause a reaction at 4°C for 60 minutes.

[0347] After the cells were washed with SM, Goat F(ab')₂ Anti-Human IgG PE (γchain specific) (Southern Biotech Inc.) diluted 500 times with SM was added to cause a reaction at 4°C for 60 minutes. The cells were washed with SM and resuspended with 50 µL of SM, and the fluorescent intensity was measured by flow cytometry (BD Biosciences, FACS Cantoll).

[0348] The data was analyzed by FLOWJO 7.65 (Tomy Digital Biology Co., Ltd.), curve fitting of the Geomean value in each concentration was performed using a Logistic curve, and the 50% effective concentration (EC₅₀) value of the binding of chLym2 and each humanized Lym2 antibody and the standard error (SE) value thereof were calculated using R language (Ver. 3.02) which is a language for statistical analysis. The results are listed in Table 1.

[Table 1]

No.	mAb	EC ₅₀ value (µg/mL)
1	chLym2	0.54 ± 0.072
2	LV0HV0	0.87 ± 0.052
3	LV1HV0	0.69 ± 0.057
4	LV2aHV0	0.66 ± 0.053
5	LV2bHV0	0.67 ± 0.061
6	LV2cHV0	0.68 ± 0.071
7	LV3aHV0	0.70 ± 0.078
8	LV3bHV0	0.81 ± 0.050
9	LV4HV0	0.74 ± 0.076

(continued)

No.	mAb	EC ₅₀ value (μg/mL)
10	LV0HV4	0.58 ± 0.052
11	LV1HV4	0.58 ± 0.045
12	LV2aHV4	0.51 ± 0.068
13	chLym2	0.50 ± 0.077
14	LV2bHV4	0.59 ± 0.038
15	LV2cHV4	0.54 ± 0.070
16	LV3aHV4	0.76 ± 0.135
17	LV3bHV4	0.53 ± 0.095
18	LV4HV4	0.68 ± 0.116
19	LV0HV0	0.55 ± 0.052
20	LV0HV1	0.78 ± 0.088
21	LV0HV2a	0.65 ± 0.077
22	LV0HV2b	0.72 ± 0.064
23	LV0HV3	0.69 ± 0.032
24	LV0HV4	0.67 ± 0.023

[0349] As the result, it was suggested that various humanized antibodies have comparable human CRTH2 reactivity to the chimeric antibody as listed in Table 1.

[Example 8]

Eosinophils and basophils depletion activity of chLym2 and humanized Lym2 antibody

[0350] Human peripheral blood collected by adding a heparin sodium injection liquid was centrifuged at 4°C and 1500 rpm for 30 minutes, and the plasma was recovered.

[0351] After the plasma was recovered, PBS (NACALAI TESQUE, Inc.) was added to the pellet containing the red blood up to the original volume of blood and the pellet was suspended. Next, to 1 mL of the suspension, 10 mL of hemolyzing solution [solution obtained by diluting 10×RBC Lysis buffer (eBioscience) 10 times with sterile water] was added to be mixed by inversion. The resultant was allowed to stand at room temperature for 10 minutes and centrifuged at room temperature and 1500 rpm for 5 minutes and washed twice with PBS after the supernatant was removed therefrom.

[0352] Thereafter, the cell pellet was suspended with the recovered plasma and the cells were seeded in a 48-well plate at 300 μL/well, each of chLym2, humanized Lym2 antibodies LV0HV0, LV0HV1, and LV0HV2a, or an isotype control antibody [IgG1 antibody prepared according to the method described in Example 5 using a vector encoding an anti-2,4-dinitrophenol (DNP) IgG1 antibody described in Clin Cancer Res 2005, 11(8), 3126 to 3135 (hereinafter, referred to as anti-DNP IgG1 antibody)] were added at each final concentrations of 1000, 100, 33, 11, 3.7, 1.2, 0.4, and 0.01 ng/mL to cause a reaction in a 5% CO₂ incubator at 37°C for 20 hours.

[0353] After the reaction, the cell fluid in each well was recovered, 10 mL of SM was added thereto, 200 μL per sample of CountBright Absolute Counting Beads, for flow cytometry (Molecular Probes) was added as control beads, and the fluid was centrifuged at 4°C and 2000 rpm for 10 minutes, and then the supernatant was removed. The resultant was washed twice with SM, 10000 μg/mL of IgG from human serum (Sigma-Aldrich Co. LLC) diluted with SM was added at 300 μL/sample and suspended to cause a reaction at 4°C for 30 minutes.

[0354] Thereafter, the cells were seeded in a 96-well plate such that each well has 40 μL of suspension and PE anti-human Siglec-8 Antibody (Biolegend Inc.), for detecting eosinophils, and PE-Cy7 Mouse Anti-Human CD123 (BD Biosciences) and Anti-Human Fc epsilon Receptor I alpha (FcεR1) APC (eBioscience), for detecting basophils, were respectively added thereto at 5 μL/well to cause a reaction at 4°C for 40 minutes.

[0355] The cells were washed twice with SM, suspended by SM containing 1% of 7-AAD Staining Solution (BD Biosciences), allowed to stand at 4°C for 10 minutes, and the fluorescent intensity was analyzed by flow cytometry (BD

Biosciences, FACS Cantoll).

[0356] Human eosinophils were detected as 7-AAD negative siglec8-PE positive fractions in granulocyte fractions of FSC-SSC deployment. Human basophils were detected as 7-AAD negative fractions, CD123-PC-Cy7 positive fractions, FcεRI-APC positive fractions in lymphocyte fractions of FSC-SSC deployment. The cell depletion activity was evaluated by analyzing the count number of each cell per a certain number of CountBright.

[0357] As the result, it was found that all evaluated humanized Lym2 antibodies LV0HV0, LV0HV1, and LV0HV2a show cytotoxic activity against eosinophils and basophils comparable to the chimeric Lym2 antibody chLym2 as shown in Figs. 3(A) to 3(C).

[Example 9]

Preparation of anti-human CRTH2 antibody for control

(1) Preparation of anti-human CRTH2 antibody expression vector for control

[0358] The base sequences encoding the amino acid sequences of VH and VL of anti-human CRTH2 monoclonal antibodies hu19A2 v52, hu8B1 v1, mu8B1, mu3C12, and mu31A5 described in Pamphlet of International Publication No.2014/144865 (respectively represented by SEQ ID NOS: 57 and 40, 64 and 52, 62 and 50, 63 and 51, and 65 and 53 in Pamphlet of International Publication No.2014/144865) were entirely synthesized.

[0359] The above described base sequences were incorporated in the antibody expression vectors so that each vector have the combinations of VH and VL of each antibody according to the method described in Example 5-(1) to prepare 5 types of humanized or chimeric anti-human CRTH2 antibody (respectively, hu19A2 v52, hu8B1 v1, ch8B1, ch3C12, and ch31A5) expression vectors for control were prepared.

(2) Preparation of anti-human CRTH2 antibody transiently expressing cells for control and purification of antibody

[0360] According to the method described in Examples 5-(2) and (3), a host cell was allowed to transiently express antibody expression vectors of hu19A2 v52, hu8B1 v1, ch8B1, ch3C12, or ch31A5, and then each antibody was purified from the culture supernatant.

[Example 10]

Epitope analysis of anti-human CRTH2 monoclonal antibody using cells which express mutated human CRTH2 with amino acid substitution

(1) Preparation of mutated human CRTH2 with amino acid substitution expression vector

[0361] A mutant with amino acid substitution expression vector obtained by partially substituting amino acid residues in an extracellular region with other amino acid residues among the amino acid sequences of human CRTH2 was prepared. Specifically, mutant with amino acid substitution expression vectors obtained by substitution of S2A; N4A; T6A and L7A; K8A, P9A, and L10A; P12A, L14A, and E15A; Q16E, R19H, and Q21R; H23A, S24A, and N25A; T26A, S27A, and I28A; D171A, T172A, and I173A; S174A, R175A, and L176A; D177A, G178A, and R179A; I180A and M181A; Y183A, Y184A, and N185A; L187A, L188A, and L189A; N190A; P191A; G192A; P193A; D194A; R195A; D196A, and T198A; N275A, G277A, and L278A; P276A; P279A and L281A; P280A; or V282A, R283A, and R284A in the amino acid sequences represented by SEQ ID NO: 2 were respectively prepared. It should be noted that the above symbols in the amino acid substitution indicate [single letter code of an amino acid residue before substitution] [substitution position counting from the N terminal] [single letter code of the amino acid residue after substitution].

[0362] Azami-Green tag was added to the intercellular C terminal region by inserting genes encoding various mutants with amino acid substitution obtained by deleting termination codons to the restriction enzyme sites of BamHI and HindIII using phmAG1-MNLinker (MBL) as expression vectors.

[0363] The above-described vectors expressing mutants with amino acid substitution were prepared using 1) a method of entirely synthesizing gene sequences encoding mutants with amino acid substitution and inserting the gene sequences into a vector or 2) a method of performing site-specific mutation from a vector into which DNA which encodes the amino acid sequences of wild-type human CRTH2 represented by SEQ ID NO: 1 was inserted.

(2) Establishment of mutated human CRTH2 with amino acid substitution transiently expressing cells

[0364] CHO-S cells (Lifetechnologies) were used for preparing mutated human CRTH2 with amino acid substitution

expressing cell lines. Free style CHO expression medium (Invitrogen) containing 8 mM L-Glutamine (Invitrogen) was used for subculturing cells and the cells were cultured by shaking under the conditions of 37°C in the presence of 5% CO₂.

[0365] 25 µg of the mutated human CRTH2 with amino acid substitution expression vector prepared in (1) described above was dissolved in 400 µL of Opti-Pro SFM (Invitrogen) and 25 µL of a Freestyle MAX Reagent (Invitrogen) was dissolved in 400 µL of Opti-Pro SFM and each of them were allowed to stand at room temperature for 5 minutes. The two liquids were mixed with each other and allowed to stand at room temperature for 15 minutes. The mixed solution was added to the CHO-S culture solution and the cells were cultured for 24 hours, thereby obtaining cell lines which express mutated human CRTH2 with amino acid substitution.

(3) Analysis of reactivity of obtained antibody using cells which express mutated human CRTH2 with amino acid substitution

[0366] The CHO-S cells which express mutated human CRTH2 with amino acid substitution established in (2) were washed with SM, seeded in a 96-well plate such that one well has 2×10^5 cells, and centrifuged at 1700 rpm for 2 minutes. After the supernatant was removed, chLym2, LV0HV1, the anti-human CRTH2 antibody hu19A2 v52, hu8B1 v1, ch3C12, or ch31A5 prepared in Example 9, or a commercially available anti-human CRTH2 antibody BM16 (Santa Cruz) prepared to have a concentration of 10 µg/mL using SM was added thereto to cause a reaction at 4°C for 1 hour.

[0367] After the cells were washed, goat anti-Human IgG Alexa 647 (Molecular Probes) diluted with SM was added to wells to which chLym2, LV0HV1, hu19A2 v52, hu8B1 v1, ch3C12, or ch31A5 was added and goat anti-Rat IgG Alexa 647 (Molecular Probes) diluted with SM was added to wells to which BM16 was added at a final concentration of 10 µg/mL to cause a reaction at 4°C for 1 hour.

[0368] After the reaction, the cells were washed and suspended with SM, and the fluorescent intensity was analyzed by flow cytometry (BD Biosciences, FACS Cantoll). At the time of analysis, positive cells of Azami-Green were subjected to gating from the whole cells and the reactivity of each antibody to this population was analyzed.

[0369] Moreover, in order to correct the expression level between mutants, the relative fluorescent intensity was calculated by dividing the fluorescent intensity of Alexa647 resulting from the binding of each antibody by the fluorescent intensity of Azami-Green added to the C terminal region and set as the reactivity of each antibody. Then, the relative fluorescent intensity regarding the cells which express mutated human CRTH2 with amino acid substitution were analyzed for each antibody when the fluorescent intensity of each antibody with respect to the wild-type human CRTH2-expressing cells was set to 100%.

[0370] As the result, it was found that the reactivity of humanized Lym2 antibody LV0HV1 against the cells which express mutated human CRTH2 with amino acid substitution of P12A, L14A, and E15A; D177A, G178A, and R179A; I180A and M181A; Y183A, Y184A, and N185A; L187A, L188A, and L189A; G192A; D194A; R195A; or D196A and T198A was lost as shown in Figs. 4 to 7.

[0371] In addition, it was found that the reactivity of the chimeric Lym2 antibody chLym2 against cells which express mutated human CRTH2 with amino acid substitution of D171A, T172A, and I173A was additionally lost. Since the reactivity of the humanized Lym2 antibody LV0HV1 against the cells which express mutated human CRTH2 with amino acid substitution containing D171A, T172A, and I173A was greatly decreased, it was suggested that both of LV1HV0 and cLym2 bind to the same epitope.

[0372] On the other hand, since the reactivity of other anti-human CRTH2 antibodies compared this time against cells which express mutated human CRTH2 with amino acid substitution at different site from the site in regard to the anti-human CRTH2 antibody of the present invention was significantly decreased, it was found that other existing anti-human CRTH2 antibodies recognize epitopes different from the antibodies of the present invention.

[0373] In addition, as a result of evaluating the reactivity against each cells which express mutated human CRTH2 with amino acid substitution, since the reactivity of the existing anti-human CRTH2 antibodies against cells which express mutated human CRTH2 with amino acid substitution containing G192A or D194A was not decreased and only the reactivity of the antibodies of the present invention against the cells was decreased, the anti-human CRTH2 antibody of the present invention was found to bind to an epitope comprising at least one amino acid residue of Gly192 and Asp194 of human CRTH2.

[Example 11]

Evaluation of reactivity against human eosinophils

[0374] To human peripheral blood collected by adding a heparin sodium injection liquid, the same amount of physiological saline for injection as the human peripheral blood was added thereto and mixed with each other. 15 mL of Ficoll-Paque PREMIUM 1.084 (GE Healthcare) was added to a 50 mL centrifuge tube, 30 mL of the above described human peripheral blood diluted with physiological saline was overlaid thereon, and centrifugation was performed at room tem-

perature and 1500 rpm for 30 minutes.

[0375] After the centrifugation, half the amount of the plasma layer, the mononuclear cell layer, and the Ficoll layer was removed by an aspirator and the platelet adhering to a wall between centrifuges was removed using sterilized swab. Thereafter, 27 mL of sterilized ice water was dispensed to each tube for hemolysis for 30 seconds.

[0376] Next, 3 mL of an ice-cold 10×PIPES buffer solution [a solution obtained by dissolving 32.15 g of sodium chloride (Wako Pure Chemical Industries), 1.85 g of potassium chloride (NACALAI TESQUE, Inc.), 38 g of PIPES (piperazine-1,4-bis(2-ethanesulfonic acid, NACALAI TESQUE, Inc.), and 8.4 g of sodium hydroxide (NACALAI TESQUE, Inc.) in 500 mL of distilled water] was added thereto to bring back to isotonicity and the resultant was centrifuged at 4°C and 1200 rpm for 5 minutes. After the centrifugation, the supernatant was aspirated, 2 mL of 1×PIPES buffer solution [a solution obtained by diluting the 10×PIPES buffer solution 10 times with distilled water] was added, and then the cell pellet was loosened.

[0377] The hemolysis operation was repeated again and the obtained cell pellet was washed twice with a solution obtained by adding a MACS BSA Stock Solution (Miltenyi Biotec) to an autoMACS Rinsing Solution (Miltenyi Biotec) (hereinafter, abbreviated as a MACS buffer).

[0378] After the number of cells was counted, eosinophils were isolated by a negative selection method using CD16 MicroBeads, human (Miltenyi Biotec) according to the attached instructions.

[0379] The isolated cells were washed with SM, seeded in a 96-well plate such that one well has 1×10^5 cells, and centrifuged at 2000 rpm for 2 minutes. After the supernatant was removed, Lym2 antibody and commercially available CRTH2 antibody BM16 (Santa Cruz), or 301108 (R&D) which were diluted with SM, were added at each final concentrations of 10, 3.3, 1.1, 0.37, and 0.12 $\mu\text{g/mL}$ to cause a reaction at 4°C for 1 hour.

[0380] After the cells were washed, CELL LAB Mouse Anti-Rat Kappa (kappa light chain specific) FITC (Beckman Coulter Inc.) diluted with SM was added to wells to which Lym2 antibody or BM16 was added and CELL LAB Goat Anti-Mouse IgG (γ chain specific) Fluorescein (FITC) Conjugate (Beckman Coulter Inc.) diluted with SM was added to wells to which 301108 was added at a final concentration of 10 $\mu\text{g/mL}$ respectively to cause a reaction at 4°C for 1 hour. The cells were washed again and suspended with SM, and the fluorescent intensity was analyzed by flow cytometry (BD Biosciences, FACS Cantoll).

[0381] As the result, it was found that the rat anti-human CRTH2 antibody Lym2 exhibits stronger reactivity against human eosinophils than commercially available rat anti-human CRTH2 antibody BM16 and mouse anti-human CRTH2 antibody 301108 (Fig. 8).

[Example 12]

Reactivity of chLym2 against human basophils

[0382] To human peripheral blood collected by adding a heparin sodium injection liquid, the same amount of physiological saline for injection as the human peripheral blood was added and mixed with each other. 15 mL of Ficoll-Paque PREMIUM 1.084 (GE Healthcare) was added to a 50mL centrifuge tube, 30 mL of the human peripheral blood diluted with physiological saline was overlaid thereon, and centrifugation was performed at room temperature and 1500 rpm for 30 minutes.

[0383] After the centrifugation, the mononuclear cell layer was recovered using a pipette and washed twice with an MACS buffer. After the number of cells was counted, basophils were isolated by a negative selection method using Basophil Isolation Kit II, human (Miltenyi Biotec) according to the attached instructions.

[0384] After the isolated cells were washed with SM, the isolated cells were seeded in a 96-well plate such that one well has 5×10^4 cells, and centrifuged at 2000 rpm for 2 minutes. After the supernatant was removed, the cells were suspended with 100 μL of SM, and chLym2 or the isotype control antibody described in Example 8 labeled using Zenon Alexa Fluor 647 Human IgG Labeling Kit (Molecular Probes) according to the attached instructions were added at a final concentration of 10 $\mu\text{g/mL}$ to cause a reaction at 4°C for 1 hour. After the cells were washed, the cells were suspended with SM, and the fluorescent intensity was analyzed by flow cytometry (BD Biosciences, FACS Cantoll).

[0385] As the result, it was found that chLym2 has reactivity against human basophils (Fig. 9). Accordingly, it was found that the chimeric anti-human CRTH2 antibody cLym2 of the present invention binds to basophils.

[Example 13]

Reactivity of humanized Lym2 antibody against human CD4-positive T-cells

[0386] In the same manner as in Example 12, a cell suspension of a mononuclear cell layer prepared using Ficoll-Paque PREMIUM (GE Healthcare) in place of Ficoll-Paque PREMIUM 1.084 was seeded in a 96-well plate at 100 $\mu\text{L/well}$, and humanized Lym2 antibody LV0HV1 which was biotin-labeled using EZ-Link Sulfo-NHS-LC-Biotin according

to the attached instructions was added, No-Weigh Format (PIERCE) at a final concentration of 10 $\mu\text{g/mL}$ to cause a reaction at 4°C for 1 hour.

[0387] After the cells were washed, streptavidin diluted with SM at a final concentration of 10 $\mu\text{g/mL}$ and Alexa Fluor 647 conjugate (Molecular Probes), FITC anti-human CD3 Antibody (Biolegend Inc.) and CD4 Antibody, Clone SK3, PE-Conjugated (stem cell technology) in the amount described in the attached instructions were added thereto to cause a reaction at 4°C for 1 hour.

[0388] After the cells were washed again, the cells were suspended by SM, and the fluorescent intensity was analyzed by flow cytometry (BD Biosciences, FACS Cantoll). Analysis was carried out by showing the fluorescent intensity of fluorescent staining resulting from humanized Lym2 antibody LV0HV1 on the vertical axis and the fluorescent intensity of fluorescent staining resulting from the CD4 antibody on the horizontal axis with respect to a cell group fractionated lymphocytes using FSC-SSC deployment and fractionated by CD3 positive cells and CD4 positive cells. In this manner, the percentage of fractions in the CD3-positive CD4-positive cells to which the humanized Lym2 antibody LV0HV1 reacted was calculated. As the result, humanized Lym2 antibody LV0HV1 reacted to approximately 2% to 3% of T cell fractions of the CD3-positive CD4-positive cells (Fig. 10).

[Example 14]

Cytotoxic activity against eosinophils and basophils

[0389] A cell suspension prepared in the same manner as in Example 8 was seeded in a 96-well plate at 95 $\mu\text{L/well}$, test antibodies were added thereto at each of final concentrations of 1000, 10, 3, 1, 0.3, and 0.1 ng/mL to cause a reaction in a 5% CO₂ incubator at 37°C for 20 hours. As the test antibodies, humanized Lym2 antibody LV0HV1, and the anti-human CRTH2 antibody hu19A2 v52, hu8B1 v1, ch3C12, and ch31A5 prepared in Example 9 were used. The anti-DNP IgG1 antibody was used as an isotype control antibody.

[0390] 100 μL of SM was respectively added to each well after the reaction, 20 μL of CountBright Absolute Counting Beads, for flow cytometry (Molecular Probes) was added to each well as control beads, and the resultant was centrifuged at 4°C and 2000 rpm for 2 minutes, and then the supernatant was removed. The resultant was washed twice with SM, 10000 $\mu\text{g/mL}$ of IgG from human serum (Sigma-Aldrich Co. LLC) diluted with SM was added at 100 $\mu\text{L/well}$ to cause a reaction at 4°C for 30 minutes. The rest was carried out in the same manner as in Example 8.

[0391] As the result, both eosinophils and basophils were removed by the humanized anti-human CRTH2 antibody LV0HV1 in an antibody concentration-dependent manner. In a case of existing anti-human CRTH2 antibody ch3C12, the activity of removing the cells was weaker than the activity of humanized anti-human CRTH2 antibody LV0HV1 [Figs. 11(A) and 11(B)]. Therefore, it is suggested that the anti-human CRTH2 antibody of the present invention can exhibit therapeutic effects targeting eosinophils or basophils which express CRTH2.

[Example 15]

Cytotoxic activity against Th2 cells

[0392] The mononuclear cell layer prepared in the same manner as in Example 13 was washed twice with RPMI-1640 (Wako Pure Chemical Industries) to which 1/10 volume FBS (GIBCO Inc.), 200 mM-L-Glutamine Stock Solution (NACAL TESQUE, Inc.), 10 mM MEM Non-Essential Amino Acids Solution (GIBCO Inc.), 100 mM Sodium Pyruvate (GIBCO Inc.), 1 M HEPES Buffer Solution (GIBCO Inc.), and 1/100 volume Penicillin-Streptomycin, Liquid (GIBCO Inc.) were added [hereinafter, referred to as peripheral blood mononuclear cell (PBMC) culture medium], thereby preparing a 1×10^7 cells/mL cell suspension.

[0393] The prepared cell suspension was seeded in a 96-well plate at 100 $\mu\text{L/well}$, chLym2 antibody, the humanized anti-human CRTH2 antibody LV0HV1 or the isotype control antibody described in Example 8 was added thereto at a final concentration of 10 $\mu\text{g/mL}$ to cause a reaction in a 5% CO₂ incubator at 37°C for 20 hours.

[0394] The cells after the reaction were washed 7 times with a PBMC culture medium to remove the test antibodies thoroughly. Thereafter, the cells were suspended with 100 μL of PBMC culture medium and seeded in a plate in which anti-CD3 antibody OKT3 (Abcam plc.) was immobilized at a concentration of 1 $\mu\text{g/mL}$. Next, an anti-CD28 antibody (BD Biosciences) prepared to have a concentration of 2 $\mu\text{g/mL}$ was added thereto at 100 $\mu\text{L/well}$ to cause a reaction in a 5% CO₂ incubator at 37°C for 3 days.

[0395] The supernatant after the reaction was recovered, and cytokine in the supernatant was quantified using Human IFN- γ Flex Set (BD Biosciences), Human IL-5 Flex Set (BD Biosciences), and Human IL-13 Flex Set (BD Biosciences) according to the attached instructions.

[0396] As the result, production of IL-5 and IL-13 which are Th2 cytokine was reduced by the chimeric anti-human CRTH2 antibody chLym2 and the humanized anti-human CRTH2 antibody LV0HV1, but production of IFN- γ which is

Th1 cytokine was not changed. In other words, it was suggested that the antibodies of the present invention selectively remove Th2 cells [Figs. 12(A) and 12(B)]. Therefore, it was suggested that the anti-human CRTH2 antibody of the present invention was able to exhibit therapeutic effects targeting the Th2 cells.

[Example 16]

Evaluation of reactivity in presence of ligand (293 EBNA)

[0397] Human CRTH2-expressing 293 EBNA cells were established by introducing the human CRTH2 gene expression pAMoh vector prepared in Example 1-(1)-(ii) into 293 EBNA cells (Invitrogen) using Fugene 6 (Promega Corporation) and selecting drug-resistant cells by culture medium formed of 10% FBS, 0.25 mg/mL of G418 (NACALAI TESQUE, Inc.), 100 µg/mL of penicillin, 100 U/mL of streptomycin (NACALAI TESQUE, Inc.), and 300 µg/mL of hygromycin B (Wako Pure Chemical Industries) in DMEM.

[0398] The human CRTH2-expressing 293 EBNA cells were peeled using 0.02% EDTA solution (NACALAI TESQUE, Inc.), washed with PBS, and suspended with the above-described culture medium. Next, the cells were seeded in a 96-well plate at 1×10^5 cells/90 µL/well and DKPGD2 (Cayman Chemical) was added thereto at a final concentration of 10 µM.

[0399] After the plate was allowed to stand in a 5% CO₂ incubator at 37°C for 15 minutes, the humanized Lym2 antibody LV0HV1, the known anti-human CRTH2 antibody hu19A2 v52, hu8B1 v1, ch3C12, ch31A5, BM16 (Santa Cruz), or 301108 (R&D) at each of final concentrations of 0.3, 1, and 3 µg/mL were added thereto to cause a reaction at room temperature for 30 minutes.

[0400] After the cells were washed 5 times with SM, 10 µg/mL of goat anti-Human IgG Alexa 647 (Molecular Probes) diluted with SM was added to wells to which LV0HV1, hu19A2 v52, hu8B1 v1, ch3C12, or ch31A5 was added, 10 µg/mL of goat anti-Rat IgG Alexa 647 (Molecular Probes) diluted with SM was added to wells to which BM16 was added, and 10 µg/mL of goat anti-Mouse IgG Alexa 647 (Molecular Probes) diluted with SM was added to wells to which 301108 was added at a final concentration of 10 µg/mL to cause a reaction at 4°C for 40 minutes. After the cells were washed again, the cells were suspended with SM, and the fluorescent intensity was analyzed by flow cytometry (BD Biosciences, FACS Cantoll).

[0401] As the result, in a case of existing anti-human CRTH2 antibodies, binding to human CRTH2-expressing cells decreased in the presence of DKPGD2 which is a human CRTH2 ligand as shown in Figs. 13(A) to (C). On the other hand, in a case of the humanized Lym2 antibody LV0HV1, binding to the human CRTH2-expressing cells was almost not decreased even at any of the antibody concentrations examined.

[0402] That is, it was found that the antibodies of the present invention exhibit high reactivity even in the presence of high concentration of the ligand. Therefore, the anti-human CRTH2 antibody of the present invention can bind to the human CRTH2-expressing cells even in the presence of a ligand with the reactivity comparable to the reactivity in the absence of a ligand and this suggests that the anti-human CRTH2 antibody of the present invention is a useful antibody which can act on the human CRTH2-expressing cells.

[Example 17]

Analysis of reactivity against differentiation-induced human mast cells

[0403] Mast cells were prepared according to the method described in Nature Protocols 2006, 1 (4), 2178 to 2183. The mast cells after 11 weeks from the start of differentiation induction were washed with SM and seeded in a 96-well plate such that each well had 5×10^4 cells, and PE anti-human CD203c (E-NPP3) Antibody (Biolegend Inc.), Brilliant Violet 421 (trademark) anti-human CD117 (c-kit) Antibody (Biolegend Inc.), and Anti-Human Fc epsilon Receptor I alpha (FcεR1) PE (eBioscience) were added thereto to cause a reaction at 4°C for 1 hour. After the cells were washed again, the cells were suspended with SM, and the fluorescent intensity was analyzed by flow cytometry (BD Biosciences, FACS Cantoll).

[0404] As the result, it was confirmed that the differentiation-induced mast cells expressed all of CD203c, CD117, and FcεRI which are mast cell surface markers.

[0405] Human IgE obtained by recombining a constant region of the anti-DNP IgG1 antibody with IgE type was added to the mast cell culture solution after 15 weeks from the start of differentiation induction at a final concentration of 10 µg/mL to cause a reaction in a 5% CO₂ incubator at 37°C for 3 days.

[0406] The mast cells after the reaction were washed twice with IMDM (GIBCO Inc.) to which 100 µg/mL of penicillin, 100 U/mL of streptomycin (NACALAI TESQUE, Inc.), and 55 µM β-ME (GIBCO Inc.) were added (hereinafter, abbreviated as a basic culture medium) and suspended with 4 mL of basic culture medium, and rabbit polyclonal anti human IgE antibody (Dako) at a final concentration of 10 µg/mL was added thereto, and then the suspension was allowed to rotate using MACS mix tube rotator (Miltenyi Biotec) to cause a reaction in a 5% CO₂ incubator at 37°C for 1 hour.

[0407] The cells after the reaction were washed twice with SM, seeded in a 96-well plate such that each well had 5×10^4 cells, and centrifuged at 2000 rpm for 2 minutes. The supernatant was removed, 10000 $\mu\text{g/mL}$ of IgG from human serum (Sigma-Aldrich Co. LLC) diluted with SM was added at 100 $\mu\text{L/well}$ to cause a reaction at 4°C for 30 minutes, and humanized anti-human CRTH2 antibody LV0HV1, anti-human CRTH2 antibody hu19A2 v52, ch8B1, ch3C12, or ch31A5, each of which was biotin-labeled using EZ-Link Sulfo-NHS-LC-Biotin, No-Weigh Format (PIERCE) according to the attached instructions, or non-labeled commercially available anti-human CRTH2 antibody BM16 (Santa Cruz Biotechnology), or 301108 (R&D) was added thereto at a final concentration of 10 $\mu\text{g/mL}$ to cause a reaction at 4°C for 1 hour.

[0408] It should be noted that Purified Rat IgG2a, κ Isotype Ctrl Antibody (BioLegend Inc.) was used for BM16, Negative Control Mouse IgG2a (Dako) was used for 301108, and the anti-DNP IgG1 antibody was used for other antibodies, as isotype control antibodies.

[0409] After the cells were washed, streptavidin, Alexa Fluor 647 conjugate (Molecular Probes) diluted with SM was added to wells to which biotin-labeled LV0HV1, hu19A2 v52, ch8B1 v1, ch3C12, or ch31A5 was added, goat anti-Rat IgG Alexa 647 (Molecular Probes) diluted with SM was added to wells to which BM16 was added, and goat anti-Mouse IgG Alexa 647 (Molecular Probes) diluted with SM was added to wells to which 301108 was added at a final concentration of 10 $\mu\text{g/mL}$ to cause a reaction at 4°C for 40 minutes. After the cells were washed again, the cells were suspended with SM, and the fluorescent intensity was analyzed by flow cytometry (BD Biosciences, FACS Cantoll).

[0410] As the result, as shown in Fig. 14, hu19A2 v52 exhibited reactivity against mast cells, but antibodies other than hu19A2 v52 (including LV0HV1) did not exhibit reactivity.

[Example 18]

Analysis of reactivity against differentiation-induced Th1

(1) Separation of naive T cells from PBMC

[0411] Healthy person-derived frozen PBMC (Allcells, LLC.) was washed once with 20 mL of PBMC culture medium containing 50 μL of DNase I recombinant, RNase-free (Roche Diagnostics K.K.), suspended with 20 mL of PBMC culture medium containing 50 μL of DNase I recombinant, RNase-free, and allowed to stand in a 5% CO_2 incubator at 37°C for 2 hours.

[0412] Thereafter, the cells were washed twice with the MACS buffer described in Example 11 and naive CD4-positive T-cells were isolated by a negative selection method using Naive CD4⁺ T Cell Isolation Kit II human (Miltenyi Biotec) according to the attached instructions. The negative selection was performed twice in order to improve purity of the naive CD4-positive T-cells.

(2) Differentiation of Th1 cells from naive CD4-positive T-cells

[0413] According to the method described in J Immunol, 2002. 169(5): p. 2498 to 2506, the naive CD4-positive T-cells separated were suspended with RPMI1640 (GIBCO Inc.) to which 10% FBS (GIBCO Inc.) and 2 mM glutamic acid (NACALAI TESQUE, Inc.) were added (hereinafter, abbreviated as a culture medium) at 1×10^6 cells/mL and seeded in a plate in which anti-CD3 antibody OKT3 (Abcam plc.) was immobilized at a concentration of 1 $\mu\text{g/mL}$, and anti-CD28 antibody (BD Biosciences) at a final concentration of 2 $\mu\text{g/mL}$, recombinant human IL-12 (PeproTech Inc.) at a final concentration of 100 ng/mL, recombinant human IL-2 (PeproTech Inc.) at a final concentration of 10 ng/mL, and anti-IL-4 antibody (BD Biosciences) at a final concentration of 5 $\mu\text{g/mL}$ were added thereto to cause a reaction in a 5% CO_2 incubator at 37°C for 4 days as a stimulation process.

[0414] Thereafter, the cells were recovered, the supernatant was removed by centrifugation, and as a growth process, the cells were suspended with of culture medium up to 1×10^6 cells/mL, and then recombinant human IL-12 (PeproTech Inc.) at a final concentration of 100 ng/mL, recombinant human IL-2 (PeproTech Inc.) at a final concentration of 10 ng/mL, and an anti-IL-4 antibody (BD Biosciences) at a final concentration of 5 $\mu\text{g/mL}$ were added thereto to cause a reaction in a 5% CO_2 incubator at 37°C for 3 days.

[0415] The differentiation-induced Th1 cells were established by repeating the stimulation process and the growth process three times.

(3) Analysis of cytokine production from differentiation-induced Th1 cells

[0416] Phorbol 12-myristate 13-acetate (Sigma-Aldrich Co. LLC) at a final concentration of 20 ng/mL, Ionomycin calcium salt from Streptomyces (Sigma-Aldrich Co. LLC) at a final concentration of 1 ng/mL, and Brefeldin A from Penicillium brefeldianum (Sigma-Aldrich Co. LLC) at a final concentration of 10 $\mu\text{g/mL}$ were added to the culture solution

containing differentiation-induced Th1 cell prepared in (2) to cause a reaction in a 5% CO₂ incubator at 37°C for 6 hours. Thereafter, the cells were seeded in a 96-well plate at 2×10^5 cells/well and washed with PBS (NACALAI TESQUE, Inc.).

[0417] Next, the dead cells were stained using LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation (Molecular Probes) according to the attached instructions and washed twice with PBS, the supernatant was removed and a reaction using a Fixation Buffer (BD Biosciences) was performed at 4°C for 45 minutes.

[0418] After the reaction, the cells were washed once with PBS and twice with $1 \times$ Perm/Wash buffer (BD Biosciences), and a PE anti-human IFN- γ Antibody (Biolegend Inc.) at a final concentration of 2.5 μ L, an APC anti-human IL-4 Antibody (Biolegend Inc.) at a final concentration of 5 μ L, anti-IL-5 antibodies APC (Miltenyi Biotec) at a final concentration of 10 μ L, and an APC anti-human IL-13 Antibody (Biolegend Inc.) at a final concentration of 20 μ L were added thereto to cause a reaction at 4°C for 30 minutes.

[0419] Thereafter, the cells were washed twice with $1 \times$ Perm/Wash buffer and suspended with SM, and the fluorescent intensity was analyzed by flow cytometry (BD Biosciences, FACS Cantoll).

[0420] As the result, it was confirmed that the differentiation-induced Th1 was a cell population in which IL-4/5/13 was negative and 90% thereof was IFN γ positive and it produced Th1 cytokine selectively.

(4) Analysis of reactivity of anti-human CRTH2 antibody against differentiation-induced Th1 cells

[0421] The differentiation-induced Th1 cells established in (2) were washed with SM, seeded in a 96-well plate such that one well has 5×10^4 cells, and centrifuged at 2000 rpm for 2 minutes. After the supernatant was removed, the rat anti-human CRTH2 antibody Lym2, a known anti-human CRTH2 antibody BM16 (Santa Cruz Biotechnology), or 301108 (R&D), each of which were diluted with SM, was added thereto at 10 μ g/mL to cause a reaction at 4°C for 1 hour.

[0422] Rat IgG2b, kappa monoclonal [RTK4530]-BSA/Azide free (Abeam plc.) was used for Lym2, Purified Rat IgG2a, κ Isotype Ctrl Antibody (BioLegend Inc.) was used for BM16, and Negative Control Mouse IgG2a (Dako) was used for 301108, as isotype control antibodies.

[0423] After the cells were washed, 10 μ g/mL of CELL LAB Mouse Anti-Rat Kappa (kappa light chain specific) FITC (Beckman Coulter Inc.) diluted with SM was added to wells to which Lym2 antibody or BM16 was added, 10 μ g/mL of CELL LAB Goat Anti-Mouse IgG (γ chain specific) Fluorescein (FITC) Conjugate (Beckman Coulter Inc.) diluted with SM was added to wells to which 301108 was added at a final concentration of 10 μ g/mL to cause a reaction at 4°C for 1 hour. After the cells were washed again, the cells were suspended with SM, and the fluorescent intensity was analyzed by flow cytometry (BD Biosciences, FACS Cantoll).

[0424] As the result, the rat anti-human CRTH2 antibody Lym2 and the commercially available rat anti-human CRTH2 antibody BM16 did not exhibit reactivity against the differentiation-induced Th1 as shown in Fig. 15. On the other hand, the commercially available mouse anti-human CRTH2 antibody 301108 exhibited reactivity against the differentiation-induced Th1.

[0425] Therefore, it was found that the anti-human CRTH2 antibody of the present invention reacts against eosinophils, basophils, and Th2 cells, but it doesn't react against Th1 cells. Further, it was found that 301108 non-specifically exhibited reactivity against cells other than the CRTH2-expressing cells.

[Example 19]

Evaluation of antagonist activity of Lym2 antibody using shape change of human eosinophils as index

[0426] Eosinophils were separated from human peripheral blood in the same manner as in Example 11. The isolated human eosinophils were suspended with Dulbecco's phosphate buffered saline (hereinafter, referred to as D-PBS (-)) (NACALAI TESQUE, Inc.) and performed a reaction in a 5% CO₂ incubator at 37°C for 1 hour and 30 minutes. Thereafter, the cells were seeded in a 96-well plate at 2×10^5 cells/well using 10% FBS-containing RPMI1640. Next, an isotype control antibody [CELL LAB Rat IgG2b Isotype Control (Beckman Coulter Inc.)] at a final concentration of 10 μ g/mL or Lym2 antibody; or small molecular CRTH2 antagonist OC000459 (Cayman Chemical) at a final concentration of 10 μ M as a positive control, was added thereto to cause a reaction in a 5% CO₂ incubator at 37°C for 30 minutes.

[0427] Next, DKPGD2 (Cayman Chemical) at each of final concentrations of 0.1, 1, 10, 100, and 1000 nM was added thereto to cause a reaction in a 5% CO₂ incubator at 37°C for 60 minutes. After the reaction, the cells were washed twice with ice-cold PBS, Fixation Buffer (BD Biosciences) was added at 200 μ L/well, and the resultant was suspended well and allowed to stand at 4°C for 30 minutes. After that, the cells were washed twice with SM, and shape change of eosinophils was analyzed by flow cytometry (BD Biosciences, FACS Cantoll).

[0428] The shape change of eosinophils was analyzed based on an increase in Forward Scatter Light (FSC) plot which is an index of the cell size, the cell surface area, or the cell diameter in the analysis using flow cytometry and the percentage (%) of eosinophils detected in the gate of high FSC was calculated based on the FSC of DKPGD2-untreated eosinophils.

[0429] As the result, human CRTH2 ligand DKPGD2 induced shape change of eosinophils in a concentration-dependent manner as shown in Fig. 16, but small molecular CRTH2 antagonist OC000459 inhibited DKPGD2-dependent shape change of eosinophils. On the other hand, both of the anti-human CRTH2 antibody Lym2 of the present invention and the isotype control antibody didn't inhibit DKPGD2-dependent shape change of eosinophils. That is, it was suggested that the anti-human CRTH2 antibody of the present invention does not have antagonist activity.

[Example 20]

Evaluation of agonist activity of Lym2 antibody using shape change of human eosinophils as index

[0430] In the same experimental system as in Example 19 described above, rat anti-human CRTH2 antibody Lym2 at each final concentration of 0, 0.01, 0.1, 1, and 10 $\mu\text{g/mL}$ was added and a reaction was performed for 1 hour and shape change of eosinophils was analyzed.

[0431] As the result, it was shown that Lym2 antibody doesn't cause the shape change of eosinophils at a concentration of 0 to 10 $\mu\text{g/mL}$ as shown in Fig. 17. That is, it was suggested that the anti-human CRTH2 antibody of the present invention does not have agonist activity.

[Example 21]

Evaluation of agonist activity and antagonist activity, and ligand-induced signal enhancing activity of anti-human CRTH2 antibody using shape change of human eosinophils as index

[0432] Eosinophils were separated from human peripheral blood in the same manner as in Example 11. In this case, the step of adding the same amount of physiological saline for injection as the blood to the blood was omitted and the eosinophils were directly overlaid on Ficoll-Paque PREMIUM 1.084 (GE Healthcare). The isolated human eosinophils were suspended with D-PBS (-) (NACALAI TESQUE, Inc.) and a reaction was performed in a 5% CO_2 incubator at 37°C for 1 hour and 30 minutes.

[0433] Thereafter, the cells were seeded in a 96-well plate at 0.7×10^5 cells/well using 10% FBS-containing RPMI1640. Next, an isotype control antibody, the humanized Lym2 antibody LV0HV1, the anti-human CRTH2 antibody hu19A2 v52, ch8B1, ch3C12, or ch31A5 prepared in Example 9, or the commercially available anti-human CRTH2 antibody BM16 (Santa Cruz Biotechnology), or 301108 (R&D) was added thereto at a final concentration of 10 $\mu\text{g/mL}$ to cause a reaction in a 5% CO_2 incubator at 37°C for 30 minutes.

[0434] It should be noted that Purified Rat IgG2a, κ Isotype Ctrl Antibody (BioLegend Inc.) was used for BM16, Negative Control Mouse IgG2a (Dako) was used for 301108, and the anti-DNP IgG1 antibody was used for other antibodies, as isotype control antibodies.

[0435] Next, RPMI1640 containing either DKPGD2 (Cayman Chemical) or 10%FBS-was added thereto at a final concentration of 100 nM to cause a reaction in a 5% CO_2 incubator at 37°C for 60 minutes. After the reaction, the cells were washed twice with ice-cold PBS, Fixation Buffer (BD Biosciences) was added at 100 μL /well, and the resultant was suspended well and allowed to stand at 4°C for 30 minutes. The cells were washed twice with SM, and the shape change of eosinophils was analyzed in the same manner as in Example 19.

[0436] As the result, a tendency of slight shape change was observed in the case of hu19A2 v52 in regard to shape change caused by the treatment of the anti-human CRTH2 antibody in the absence of a ligand as shown in Figs. 18(A) to 18(C). However, there were no antibody which caused strong shape change and it is suggested that all antibodies do not have agonist activity.

[0437] Further, in regard to shape change caused by the treatment of anti-human CRTH2 antibody under the conditions of DKPGD2 treatment, since shape change induced by DKPGD2 was suppressed by the treatment of ch8B1, ch3C12, and ch31A5, it was suggested that these antibodies have antagonist activity. These results are consistent with the findings of the prior patent document (Pamphlet of International Publication No.2014/144865).

[0438] On the other hand, hu19A2 v52 and BM16 enhanced the shape change induced by DKPGD2. Particularly, it was found that hu19A2 v52 enhanced the shape change induced by 100 nM of DKPGD2 up to approximately twice.

[0439] As described above, since shape change is not induced by hu19A2 v52 and BM16 antibody in the absence of the ligand, it was suggested that these antibodies have signal-enhancing activity wherein the signal is caused by DKPGD2 which is a CRTH2 ligand.

[0440] On the other hand, since LV0HV1 and 301108 do not affect shape change under the both conditions of presence and absence of DKPGD2 as similar to the isotype control antibody, it was suggested that LV0HV1 and 301108 do not have signal enhancing activity wherein the signal is caused by DKPGD2.

[0441] The anti-human CRTH2 antibody of the present invention is preferable in that it does not block or enhance a physiological signal.

[Example 22]

Evaluation of reactivity of CRTH2 monoclonal antibody with respect to conformation change accompanied by transition of CRTH2 activation state

(1) Preparation of horseradish peroxidase (HRP)-labeled antibody

[0442] Humanized Lym2 antibody LV0HV1 and hu19A2 v52 were directly labeled with HRP using Peroxidase Labeling Kit-NH2 (Dojindo Molecular Technologies, Inc.) according to the attached document and diluted to 1 mg/mL with D-PBS (-) (NACALAI TESQUE, Inc.).

(2) Preparation of membrane fractions of human CRTH2-expressing cells

[0443] The human CRTH2-expressing CHO/DG44 cells established in Example 1 were peeled using 0.02% EDTA solution (NACALAI TESQUE, Inc.) and the cells were washed with D-PBS (-) cooled to 4°C. The membrane fractions were prepared using Minute Plasma Membrane Protein Isolation Kit (Invent Biotechnologies) according to the attached document.

(3) Evaluation of reactivity of anti-human CRTH2 antibody against membrane fractions of human CRTH2-expressing cells

[0444] 50 mM Tris-HCl buffer pH 7.4 (NACALAI TESQUE, Inc.) to which 50 mM HEPES (GIBCO Inc.), 5 mM MgCl₂ (NACALAI TESQUE, Inc.), 100 mM NaCl (NACALAI TESQUE, Inc.), and 1 mM EDTA (Invitrogen) were added (hereinafter, referred to as an ELISA reaction solution) was added to the membrane fractions prepared in (2) to have a concentration of 1 mg/mL, and the mixture was added to PROTEOSAVE 1.5 mL Microtubes (Sumitomo Bakelite Co., Ltd.) at 50 µL/tube.

[0445] Next, an ELISA reaction solution to which 500 µM GTPγS (Roche Diagnostics K.K.) or 500 µM GDP (Sigma-Aldrich Co. LLC) was added was added to the microtubes at 50 µL/tube for incubation at 30°C for 1 hour.

[0446] Subsequently, IgG from human serum (Sigma-Aldrich Co. LLC) diluted to 10 mg/mL with D-PBS (-) and 30% w/v BSA-PBS fatty acid free (Wako) were respectively added to the microtubes at 50 µL/tube for incubation at 30°C for 30 minutes.

[0447] Next, HRP-labeled LV0HV1 or hu19A2 v52 prepared in (1) described above was diluted to 5 µg/mL with the ELISA reaction solution and added to the microtubes at 50 µL/tube for incubation at 30°C for 1 hour. In this manner, the antibody reaction against membrane fractions was carried out. The supernatant was removed after centrifugation at 4°C for 30 minutes at 16000 g.

[0448] Thereafter, an ELISA reaction solution to which 100 µM GTPγS was added was added to tubes to which GTPγS was originally added and an ELISA reaction solution to which 100 µM GDP was added was added to tubes to which GDP was originally added respectively by 1 mL/tube. The supernatant was removed after centrifugation at 4°C for 30 minutes at 16000 g again. After the same procedure was repeated four times, D-PBS (-) was added at 100 µL/tube and membrane fractions were sufficiently suspended.

[0449] The suspension was added to a 96-well plate at 30 µL/well, and 1-Step Ultra TMB-ELISA reagent (Thermo Scientific) was added to 96-well plate at 100 µL/well. After the reaction at room temperature for 10 minutes, 0.5 mol/L of sulfuric acid (Wako) was added by 100 µL/well to stop the reaction.

[0450] The absorbance at 480 nm was measured by SPECTRA max 340PC and the obtained result was analyzed using Graphpad Prism (ver. 6.05). Further, Turkey's multiple comparisons test was adapted and performed together with a significance difference test.

[0451] As the result, the reactivity of hu19A2 v52 with respect to membrane fractions at the time of GDP treatment was significantly decreased compared to when GTPγS treatment was carried out ($P < 0.0001$) as shown in Fig 19. On the other hand, the reactivity of LV0HV1 was not changed at the time of GTPγS treatment or GDP treatment ($p > 0.1$).

[0452] It is known that CRTH2 is a GPCR and in general, GTP binds to GPCR in the activated conformation and GDP binds to GPCR in the inactivated conformation. As shown from the results described above, since the reactivity of the anti-human CRTH2 antibody of the present invention against CRTH2 was not changed regardless of the presence or absence of the treatment of GTPγS which is GTP analog or GDP, the possibility was suggested that the anti-human CRTH2 antibody is not affected by the conformation change accompanied by activation of CRTH2 and exhibits constant reactivity.

[Example 23]

Preparation of Azami-Green fusion human and cynomolgus monkey CRTH2 expression phmAG1-MNLinker vectors

(1) Preparation of Azami-Green fusion human CRTH2 expression phmAG1-MNLinker vector

[0453] The target fragment was amplified by PCR using primer human CRTH2 azami-A (SEQ ID NO: 60) and human CRTH2 azami-B (SEQ ID NO: 61) from the human CRTH2 gene expression pAMoh prepared in (1)-(ii) of Example 1 and was connected to a vector phmAG1-MNLinker (MBL) using restriction enzymes BamHI and HindIII, thereby constructing an Azami-Green fusion human CRTH2 expression phmAG1-MNLinker vector.

(2) Preparation of Azami-Green fusion cynomolgus monkey CRTH2 expression phmAG1-MNLinker vector

[0454] An azami-Green fusion cynomolgus monkey CRTH2 expression phmAG1-MNLinker vector was constructed in the same manner as in (1) using primer cyno CRTH2 azami-A (SEQ ID NO: 64) and cyno CRTH2 azami-B (SEQ ID NO: 65) from the cynomolgus monkey CRTH2 expression pMoh vector constructed in the same manner as in (1)-(ii) of Example 1 using cDNA of cynomolgus monkey CRTH2 which was entirely synthesized (cDNA sequence: SEQ ID NO: 62 and amino acid sequence: SEQ ID NO: 63).

[Example 24]

Establishment of Azami-Green fusion human CRTH2-expressing CHO/DG44 cells and cynomolgus monkey CRTH2-expressing CHO/DG44 cells

[0455] The human and cynomolgus monkey CRTH2 expression phmAG1-MNLinker vectors prepared in Example 23 was cleaved by restriction enzyme BsaI treatment and then the obtained linear DNA was purified and dissolved in sterile water. This DNA was introduced to CHO/DG44 cells by an electroporation method and cultured in IMDM culture medium for 3 days.

[0456] Thereafter, drug-resistant cells were selected by the IMDM selected medium to which 0.5 mg/mL G418 (NACALAI TESQUE, Inc.) was added. The selected drug-resistant cells were peeled using a 0.25% Trypsin-EDTA (NACALAI TESQUE, Inc.), washed with PBS, and suspended by the IMDM selected medium. Subsequently, cell populations showing Azami-Green fluorescent intensities similar to each other were gated from the human CRTH2-expressing cells and cynomolgus monkey CRTH2-expressing cells using Cell Sorter SH800 (Sony), sorted out by 1 cell/well, and expansively cultured, to establish Azami-Green fusion human CRTH2-expressing CHO/DG44 cells and cynomolgus monkey CRTH2-expressing CHO/DG44 cells.

[0457] In regard to Azami-Green fusion human CRTH2-expressing CHO/DG44 cells and cynomolgus monkey CRTH2-expressing CHO/DG44 cells, cells were prepared in the same manner as described above and Azami-Green expression was confirmed using flow cytometry (BD Biosciences, FACS Cantoll). As the result, it was confirmed that the level of Azami-Green expression was the same in each cells as shown in Fig. 20.

[Example 25]

Evaluation of binding activity of humanized Lym2 antibody LV0HV1 against human or cynomolgus monkey CRTH2

[0458] The Azami-Green fusion human CRTH2-expressing CHO/DG44 cells and the cynomolgus monkey CRTH2-expressing CHO/DG44 cells established in Example 24 were peeled using 0.02% EDTA solution (NACALAI TESQUE, Inc.), washed with PBS, and suspended with SM. Next, the cells were seeded in a 96-well plate such that each well had 2×10^5 cells, and humanized Lym2 antibody LV0HV1 or the anti-DNP IgG1 antibody at each of final concentrations 30000, 7500, 1875, 469, 117, 29, 7, and 2 ng/mL were added thereto to cause a reaction at 4°C for 40 minutes.

[0459] After the cells were washed three times with SM, goat anti-Human IgG alexa 647 (Molecular Probes) diluted to a concentration of 10 µg/mL with SM was added thereto at 100 µL/well to perform a reaction at 4°C for 40 minutes. The cells were washed with SM and resuspended with 100 µL of SM, and the fluorescent intensity was measured by flow cytometry (BD Biosciences, FACS Cantoll).

[0460] The data was analyzed by FLOWJo 7.65 (Tomy Digital Biology Co., Ltd.). As the result, it was found that the binding activity of LV0HV1 against human CRTH2 and monkey CRTH2 were substantially the same as each other as shown in Fig. 21.

Industrial Applicability

[0461] According to the present invention, it is possible to provide an anti-human CRTH2 antibody which has desired activity by recognizing and binding to a specific epitope of human CRTH2; the antibody fragment thereof; DNA which encodes the amino acid sequence of the antibody; a vector which includes the DNA; hybridomas and antibody producing cells which produce the antibody; a method of producing the antibody; a composition which includes the antibody or the antibody fragment; a treatment method and a diagnostic method of an allergic disease, an autoimmune disease, a disease accompanied by at least one of increase and hyperergasia of eosinophils; a disease accompanied by at least one of increase and hyperergasia of Th2 cells using the antibody or the antibody fragment; and a medicine and a diagnostic agent which include the antibody or the antibody fragment.

[0462] The present invention has been described with reference to particular embodiments, but various changes and modifications can be made without departing from the spirit and scope of the present invention, and this is apparent to those skilled in the art. Further, the present application is based on Japanese Patent Application No. 2015-141633 filed on July 15, 2015, the entire contents of which are incorporated herein by reference.

Sequence Listing Free Text

[0463]

SEQ ID NO: 3: description of artificial sequence: base sequence of human CRTH2 FLAG-A
 SEQ ID NO: 4: description of artificial sequence: base sequence of human CRTH2 FLAG-B
 SEQ ID NO: 5: description of artificial sequence: base sequence of FLAG-tagged human CRTH2 cDNA
 SEQ ID NO: 6: description of artificial sequence: base sequence of human CRTH2 FLAG-C
 SEQ ID NO: 7: description of artificial sequence: base sequence of human CRTH2 FLAG-D
 SEQ ID NO: 8: description of artificial sequence: base sequence of Rat IgG 2bH-A
 SEQ ID NO: 9: description of artificial sequence: base sequence of Rat IgG 2bH-B
 SEQ ID NO: 10: description of artificial sequence: base sequence of Ratk-A
 SEQ ID NO: 11: description of artificial sequence: base sequence of Ratk-B
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SEQ ID NO: 64: description of artificial sequence: base sequence of cyno CRTH2 azami-A
15 SEQ ID NO: 65: description of artificial sequence: base sequence of cyno CRTH2 azami-B

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	gtg gcc gcg gcg cac aaa gtc tgc ctg gtg ctt tgg gca cta gcg gtg	480
	Val Ala Ala Ala His Lys Val Cys Leu Val Leu Trp Ala Leu Ala Val	
	145 150 155 160	
5	ctc aac acg gtg ccc tat ttc gtg ttc cgg gac acc atc tcg cgg ctg	528
	Leu Asn Thr Val Pro Tyr Phe Val Phe Arg Asp Thr Ile Ser Arg Leu	
	165 170 175	
10	gac ggg cgc att atg tgc tac tac aat gtg ctg ctc ctg aac ccg ggg	576
	Asp Gly Arg Ile Met Cys Tyr Tyr Asn Val Leu Leu Leu Asn Pro Gly	
	180 185 190	
15	cct gac cgc gat gcc acg tgc aac tcg cgg cag gtg gcc ctg gcc gtc	624
	Pro Asp Arg Asp Ala Thr Cys Asn Ser Arg Gln Val Ala Leu Ala Val	
	195 200 205	
20	agc aag ttc ctg ctg gcc ttc ctg gtg ccg ctg gcg atc atc gcc tcg	672
	Ser Lys Phe Leu Leu Ala Phe Leu Val Pro Leu Ala Ile Ile Ala Ser	
	210 215 220	
25	agc cac gcg gcc gtg agc ctg cgg ttg cag cac cgc ggc cgc cgg cgg	720
	Ser His Ala Ala Val Ser Leu Arg Leu Gln His Arg Gly Arg Arg Arg	
	225 230 235 240	
30	cca ggc cgc ttc gtg cgc ctg gtg gcg gcc gtc gtg gcc gcc ttc gcg	768
	Pro Gly Arg Phe Val Arg Leu Val Ala Ala Val Val Ala Ala Phe Ala	
	245 250 255	
35	ctc tgc tgg ggg ccc tac cac gtg ttc agc ctg ctg gag gcg cgg gcg	816
	Leu Cys Trp Gly Pro Tyr His Val Phe Ser Leu Leu Glu Ala Arg Ala	
	260 265 270	
40	cac gca aac ccg ggg ctg cgg ccg ctc gtg tgg cgc ggg ctg ccc ttc	864
	His Ala Asn Pro Gly Leu Arg Pro Leu Val Trp Arg Gly Leu Pro Phe	
	275 280 285	
45	gtc acc agc ctg gcc ttc ttc aac agc gtg gcc aac ccg gtg ctc tac	912
	Val Thr Ser Leu Ala Phe Phe Asn Ser Val Ala Asn Pro Val Leu Tyr	
	290 295 300	
50	gtg ctc acc tgc ccc gac atg ctg cgc aag ctg cgg cgc tcg ctg cgc	960
	Val Leu Thr Cys Pro Asp Met Leu Arg Lys Leu Arg Arg Ser Leu Arg	
	305 310 315 320	
55	acg gtg ctg gag agc gtg ctg gtg gac gac agc gag ctg ggt ggc gcg	1008
	Thr Val Leu Glu Ser Val Leu Val Asp Asp Ser Glu Leu Gly Gly Ala	
	325 330 335	
60	gga agc agc cgc cgc cgc cgc acc tcc tcc acc gcc cgc tcg gcc tcc	1056
	Gly Ser Ser Arg Arg Arg Arg Thr Ser Ser Thr Ala Arg Ser Ala Ser	
	340 345 350	
65	cct tta gct ctc tgc agc cgc ccg gag gaa ccg cgg ggc ccc gcg cgt	1104
	Pro Leu Ala Leu Cys Ser Arg Pro Glu Glu Pro Arg Gly Pro Ala Arg	
	355 360 365	
70	ctc ctc ggc tgg ctg ctg ggc agc tgc gca gcg tcc ccg cag acg ggc	1152
	Leu Leu Gly Trp Leu Leu Gly Ser Cys Ala Ala Ser Pro Gln Thr Gly	
	370 375 380	
75	ccc ctg aac cgg gcg ctg agc agc acc tcg agt tag	1188
	Pro Leu Asn Arg Ala Leu Ser Ser Thr Ser Ser	
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<210> 2
 <211> 395
 <212> PRT
 <213> Homo sapiens

<400> 2

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His	Ala	Ala	Val	Leu	Leu	His	Gly	Leu	Ala	Ser	Leu	Leu	Gly	Leu	Val
		35					40					45			
Glu	Asn	Gly	Val	Ile	Leu	Phe	Val	Val	Gly	Cys	Arg	Met	Arg	Gln	Thr
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Val	Val	Thr	Thr	Trp	Val	Leu	His	Leu	Ala	Leu	Ser	Asp	Leu	Leu	Ala
65					70					75					80
Ser	Ala	Ser	Leu	Pro	Phe	Phe	Thr	Tyr	Phe	Leu	Ala	Val	Gly	His	Ser
				85					90					95	
Trp	Glu	Leu	Gly	Thr	Thr	Phe	Cys	Lys	Leu	His	Ser	Ser	Ile	Phe	Phe
			100					105					110		
Leu	Asn	Met	Phe	Ala	Ser	Gly	Phe	Leu	Leu	Ser	Ala	Ile	Ser	Leu	Asp
		115					120					125			
Arg	Cys	Leu	Gln	Val	Val	Arg	Pro	Val	Trp	Ala	Gln	Asn	His	Arg	Thr
	130					135					140				
Val	Ala	Ala	Ala	His	Lys	Val	Cys	Leu	Val	Leu	Trp	Ala	Leu	Ala	Val
145					150					155					160
Leu	Asn	Thr	Val	Pro	Tyr	Phe	Val	Phe	Arg	Asp	Thr	Ile	Ser	Arg	Leu
				165					170					175	
Asp	Gly	Arg	Ile	Met	Cys	Tyr	Tyr	Asn	Val	Leu	Leu	Leu	Asn	Pro	Gly
			180					185					190		
Pro	Asp	Arg	Asp	Ala	Thr	Cys	Asn	Ser	Arg	Gln	Val	Ala	Leu	Ala	Val
		195					200					205			
Ser	Lys	Phe	Leu	Leu	Ala	Phe	Leu	Val	Pro	Leu	Ala	Ile	Ile	Ala	Ser
	210					215					220				

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Ser His Ala Ala Val Ser Leu Arg Leu Gln His Arg Gly Arg Arg Arg
225 230 235 240

5 Pro Gly Arg Phe Val Arg Leu Val Ala Ala Val Val Ala Ala Phe Ala
245 250 255

10 Leu Cys Trp Gly Pro Tyr His Val Phe Ser Leu Leu Glu Ala Arg Ala
260 265 270

15 His Ala Asn Pro Gly Leu Arg Pro Leu Val Trp Arg Gly Leu Pro Phe
275 280 285

Val Thr Ser Leu Ala Phe Phe Asn Ser Val Ala Asn Pro Val Leu Tyr
290 295 300

20 Val Leu Thr Cys Pro Asp Met Leu Arg Lys Leu Arg Arg Ser Leu Arg
305 310 315 320

25 Thr Val Leu Glu Ser Val Leu Val Asp Asp Ser Glu Leu Gly Gly Ala
325 330 335

Gly Ser Ser Arg Arg Arg Arg Thr Ser Ser Thr Ala Arg Ser Ala Ser
340 345 350

30 Pro Leu Ala Leu Cys Ser Arg Pro Glu Glu Pro Arg Gly Pro Ala Arg
355 360 365

35 Leu Leu Gly Trp Leu Leu Gly Ser Cys Ala Ala Ser Pro Gln Thr Gly
370 375 380

40 Pro Leu Asn Arg Ala Leu Ser Ser Thr Ser Ser
385 390 395

45 <210> 3
<211> 27
<212> DNA
<213> Artificial

<220>
<223> description of the artificial sequence: human CRTH2 FLAG-A

50 <400> 3
cataagcttg ccaccatgtc ggccaac

27

55 <210> 4
<211> 66
<212> DNA
<213> Artificial

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<220>

<223> description of the artificial sequence: human CRTH2 FLAG-B

<400> 4

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gttcag 66

<210> 5

<211> 1212

<212> DNA

<213> Artificial

<220>

<223> description of the artificial sequence: FLAG tagged human CRTH2 cDNA

<400> 5

atgtcggcca acgccacact gaagccactc tgcccatcc tggagcagat gagccgtctc 60

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ctggcctcgc tgctgggcct ggtggagaat ggagtcaccc tcttcgtggt gggctgccgc 180

atgcgccaga ccgtgggtcac cacctgggtg ctgcacctgg cgctgtccga cctgttggcc 240

tctgcttccc tgcccttctt cacctacttc ttggccgtgg gccactcgtg ggagctgggc 300

accaccttct gcaaaactgca ctctccatc ttctttctca acatgttcgc cagcggcttc 360

ctgctcagcg ccatcagcct ggaccgctgc ctgcaggtgg tgcggccggt gtgggcgcag 420

aaccacgcga ccgtggccgc ggcgcacaaa gtctgcctgg tgctttgggc actagcgggtg 480

ctcaacacgg tgccctattt cgtgttccgg gacaccatct cgcggctgga cgggcgcatt 540

atgtgctact acaatgtgct gctcctgaac cgggggcctg accgcgatgc cacgtgcaac 600

tcgcggcagg tggccctggc cgtcagcaag ttctgtctgg ccttcctggt gccgtggcg 660

atcatcgcct cgagccacgc ggccgtgagc ctgcgggtgc agcaccgcgg ccgccggcgg 720

ccaggccgct tcgtgcgcct ggtggcggcc gtctgtggcc ccttcgcgct ctgctggggg 780

ccctaccacg tggtcagcct gctggaggcg cgggcgcacg caaaccggg gctgcggccg 840

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ccggtgctct acgtgctcac ctgccccgac atgctgcgca agctgcggcg ctgctgctgc 960

acggtgctgg agagcgtgct ggtggacgac agcagctgg gtggcgcggg aagcagccgc 1020

cgccgcgcga cctcctccac cgcccgctcg gcctccctt tagctctctg cagccgcccg 1080

gaggaaccgc ggggccccgc gcgtctctc ggctggctgc tgggcagctg cgcagcgtcc 1140

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gacgataagt ag 1212

<210> 6

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<211> 27
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<400> 6
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10 <210> 7
 <211> 27
 <212> DNA
 <213> Artificial

15 <220>
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<400> 7
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20 <210> 8
 <211> 24
 <212> DNA
 <213> Artificial

25 <220>
 <223> description of the artificial sequence: RatIgG2bH-A

<400> 8
 cgctggacag ggctccagag ttcc 24

30 <210> 9
 <211> 28
 <212> DNA
 <213> Artificial

35 <220>
 <223> description of the artificial sequence: RatIgG2bH-B

<400> 9
 gggcatgtag ggcatttgtg tccaatgc 28

40 <210> 10
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 <212> DNA
 <213> Artificial

45 <220>
 <223> description of the artificial sequence: Ratk-A

50 <400> 10
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55 <210> 11
 <211> 29
 <212> DNA
 <213> Artificial

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<220>

<223> description of the artificial sequence: Ratk-B

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5 cctgttgaag ctcttgacga cgggtgagg 29

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<211> 426

10 <212> DNA

<213> Rattus norvegicus

<220>

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1 5 10 15

gtc cag tgt gag gtg cag ctg gtg gag tct ggg gga ggc tta gtg cag 96
Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln
20 25 30

cct gga agg tcc atg aaa ctc tcc tgt gca gcc tca gga ttc act ttc 144
Pro Gly Arg Ser Met Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
35 40 45

agt aac tat tac atg gcc tgg gtc cgc cag gct cca aag aag ggt ctg 192
Ser Asn Tyr Tyr Met Ala Trp Val Arg Gln Ala Pro Lys Lys Gly Leu
50 55 60

gag tgg gtc gca acc att agt tat gat ggt agt agc act tac tat cga 240
Glu Trp Val Ala Thr Ile Ser Tyr Asp Gly Ser Ser Thr Tyr Tyr Arg
65 70 75 80

gac tcc gtg aag ggc cga ttc act atc tcc aga gat aat gca aaa agc 288
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Ser
85 90 95

acc cta tac ctg caa atg gac agt ctg agg tct gag gac acg gcc act 336
Thr Leu Tyr Leu Gln Met Asp Ser Leu Arg Ser Glu Asp Thr Ala Thr
100 105 110

tat tac tgt gca aga cat cgg ggt tat tac tac agt ggg gcg ggg tac 384
Tyr Tyr Cys Ala Arg His Arg Gly Tyr Tyr Tyr Ser Gly Ala Gly Tyr
115 120 125

ttt gat tac tgg ggc caa gga gtc atg gtc aca gtc tcc tca 426
Phe Asp Tyr Trp Gly Gln Gly Val Met Val Thr Val Ser Ser
130 135 140

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<210> 13

<211> 142

<212> PRT

55 <213> Rattus norvegicus

<400> 13

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5	Val	Gln	Cys	Glu	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	
				20					25					30			
	Pro	Gly	Arg	Ser	Met	Lys	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe	
10			35					40					45				
	Ser	Asn	Tyr	Tyr	Met	Ala	Trp	Val	Arg	Gln	Ala	Pro	Lys	Lys	Gly	Leu	
		50					55					60					
15	Glu	Trp	Val	Ala	Thr	Ile	Ser	Tyr	Asp	Gly	Ser	Ser	Thr	Tyr	Tyr	Arg	
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	Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Ser	
20					85					90					95		
	Thr	Leu	Tyr	Leu	Gln	Met	Asp	Ser	Leu	Arg	Ser	Glu	Asp	Thr	Ala	Thr	
25				100					105					110			
	Tyr	Tyr	Cys	Ala	Arg	His	Arg	Gly	Tyr	Tyr	Tyr	Ser	Gly	Ala	Gly	Tyr	
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30	Phe	Asp	Tyr	Trp	Gly	Gln	Gly	Val	Met	Val	Thr	Val	Ser	Ser			
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	Met	Lys	Val	Pro	Gly	Arg	Leu	Leu	Val	Leu	Leu	Phe	Trp	Ile	Pro	Ala	
	1				5					10					15		
	tcc	agg	agt	gat	gtt	gtg	ttg	aca	caa	act	cca	gtt	tcc	ctg	tct	gtc	96
	Ser	Arg	Ser	Asp	Val	Val	Leu	Thr	Gln	Thr	Pro	Val	Ser	Leu	Ser	Val	
50				20					25				30				
	aca	ctt	gga	gat	caa	gct	tct	ata	tct	tgc	agg	tct	agt	cag	agc	ctg	144
	Thr	Leu	Gly	Asp	Gln	Ala	Ser	Ile	Ser	Cys	Arg	Ser	Ser	Gln	Ser	Leu	
			35				40					45					
55	gaa	tat	agt	gat	gga	tac	act	tat	ttg	gaa	tgg	tac	cta	cag	aag	cca	192
	Glu	Tyr	Ser	Asp	Gly	Tyr	Thr	Tyr	Leu	Glu	Trp	Tyr	Leu	Gln	Lys	Pro	
		50					55					60					

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	ggc cag tct cca cag gtc ctc atc tat gga gtt tcc aac cga ttt tct	240
	Gly Gln Ser Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser	
	65 70 75 80	
5	ggg gtc cca gac agg ttc att ggc agt ggg tca ggg aca gat ttc acc	288
	Gly Val Pro Asp Arg Phe Ile Gly Ser Gly Ser Gly Thr Asp Phe Thr	
	85 90 95	
10	ctc aag atc agc aga gta gag cct gag gac ttg gga gtt tat tac tgc	336
	Leu Lys Ile Ser Arg Val Glu Pro Glu Asp Leu Gly Val Tyr Tyr Cys	
	100 105 110	
15	ttc caa gct aca cat gat cct ctc acg ttc ggc tca ggg acg aag ttg	384
	Phe Gln Ala Thr His Asp Pro Leu Thr Phe Gly Ser Gly Thr Lys Leu	
	115 120 125	
20	gaa ata aaa	393
	Glu Ile Lys	
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	<213> Rattus norvegicus	
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35	Ser Arg Ser Asp Val Val Leu Thr Gln Thr Pro Val Ser Leu Ser Val	
	20 25 30	
40	Thr Leu Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu	
	35 40 45	
45	Glu Tyr Ser Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro	
	50 55 60	
50	Gly Gln Ser Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser	
	65 70 75 80	
55	Gly Val Pro Asp Arg Phe Ile Gly Ser Gly Ser Gly Thr Asp Phe Thr	
	85 90 95	
60	Leu Lys Ile Ser Arg Val Glu Pro Glu Asp Leu Gly Val Tyr Tyr Cys	
	100 105 110	
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	115 120 125	
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<220>
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 tcc atg aaa ctc tcc tgt gca gcc tca gga ttc act ttc agt aac tat 96
 Ser Met Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
 20 25 30
 tac atg gcc tgg gtc cgc cag gct cca aag aag ggt ctg gag tgg gtc 144
 Tyr Met Ala Trp Val Arg Gln Ala Pro Lys Lys Gly Leu Glu Trp Val
 35 40 45
 gca acc att agt tat gat ggt agt agc act tac tat cga gac tcc gtg 192
 Ala Thr Ile Ser Tyr Asp Gly Ser Ser Thr Tyr Arg Asp Ser Val
 50 55 60
 aag ggc cga ttc act atc tcc aga gat aat gca aaa agc acc cta tac 240
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Ser Thr Leu Tyr
 65 70 75 80
 ctg caa atg gac agt ctg agg tct gag gac acg gcc act tat tac tgt 288
 Leu Gln Met Asp Ser Leu Arg Ser Glu Asp Thr Ala Thr Tyr Tyr Cys
 85 90 95
 gca aga cat cgg ggt tat tac tac agt ggg gcg ggg tac ttt gat tac 336
 Ala Arg His Arg Gly Tyr Tyr Tyr Ser Gly Ala Gly Tyr Phe Asp Tyr
 100 105 110
 tgg ggc caa gga gtc atg gtc aca gtc tcc tca 369
 Trp Gly Gln Gly Val Met Val Thr Val Ser Ser
 115 120

<210> 17
 <211> 123
 <212> PRT
 <213> Rattus norvegicus

<400> 17
 Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg
 1 5 10 15
 Ser Met Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
 20 25 30
 Tyr Met Ala Trp Val Arg Gln Ala Pro Lys Lys Gly Leu Glu Trp Val
 35 40 45

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	Ala Thr Ile Ser Tyr Asp Gly Ser Ser Thr Tyr Tyr Arg Asp Ser Val	
	50 55 60	
5	Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Ser Thr Leu Tyr	
	65 70 75 80	
10	Leu Gln Met Asp Ser Leu Arg Ser Glu Asp Thr Ala Thr Tyr Tyr Cys	
	85 90 95	
15	Ala Arg His Arg Gly Tyr Tyr Tyr Ser Gly Ala Gly Tyr Phe Asp Tyr	
	100 105 110	
20	Trp Gly Gln Gly Val Met Val Thr Val Ser Ser	
	115 120	
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	<211> 336	
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30	<220>	
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	<222> (1) .. (336)	
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	Asp Val Val Leu Thr Gln Thr Pro Val Ser Leu Ser Val Thr Leu Gly	
	1 5 10 15	
40	gat caa gct tct ata tct tgc agg tct agt cag agc ctg gaa tat agt 96	
	Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Glu Tyr Ser	
	20 25 30	
45	gat gga tac act tat ttg gaa tgg tac cta cag aag cca ggc cag tct 144	
	Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser	
	35 40 45	
50	cca cag gtc ctc atc tat gga gtt tcc aac cga ttt tct ggg gtc cca 192	
	Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro	
	50 55 60	
55	gac agg ttc att ggc agt ggg tca ggg aca gat ttc acc ctc aag atc 240	
	Asp Arg Phe Ile Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile	
	65 70 75 80	
60	agc aga gta gag cct gag gac ttg gga gtt tat tac tgc ttc caa gct 288	
	Ser Arg Val Glu Pro Glu Asp Leu Gly Val Tyr Tyr Cys Phe Gln Ala	
	85 90 95	
65	aca cat gat cct ctc acg ttc ggc tca ggg acg aag ttg gaa ata aaa 336	
	Thr His Asp Pro Leu Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys	
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<212> PRT
<213> Rattus norvegicus

<400> 19

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Asp Val Val Leu Thr Gln Thr Pro Val Ser Leu Ser Val Thr Leu Gly
1 5 10 15

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Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Glu Tyr Ser
20 25 30

15

Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
35 40 45

Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro
50 55 60

20

Asp Arg Phe Ile Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80

25

Ser Arg Val Glu Pro Glu Asp Leu Gly Val Tyr Tyr Cys Phe Gln Ala
85 90 95

30

Thr His Asp Pro Leu Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
100 105 110

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<213> Artificial

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<220>
<223> description of the artificial sequence: Lym2 VH CDR1 amino acid

<400> 20

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Asn Tyr Tyr Met Ala
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<210> 21
<211> 17
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<220>
<223> description of the artificial sequence: Lym2 VH CDR2 amino acid

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<400> 21

Thr Ile Ser Tyr Asp Gly Ser Ser Thr Tyr Tyr Arg Asp Ser Val Lys
1 5 10 15

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Gly

5 <210> 22
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 <213> Artificial

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 1 5 10

 15 <210> 23
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 <212> PRT
 <213> Artificial

 20 <220>
 <223> description of the artificial sequence: Lym2 VL CDR1 amino acid

 <400> 23

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 1 5 10 15

 30 <210> 24
 <211> 7
 <212> PRT
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 <220>
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 35 <400> 24

 Gly Val Ser Asn Arg Phe Ser
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 40 <210> 25
 <211> 9
 <212> PRT
 <213> Artificial

 45 <220>
 <223> description of the artificial sequence: Lym2 VL CDR3 amino acid

 <400> 25

 50 Phe Gln Ala Thr His Asp Pro Leu Thr
 1 5

 55 <210> 26
 <211> 437
 <212> DNA
 <213> Artificial

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<220>

<223> description of the artificial sequence: synthetic DNA of chLym2
VH for vector

5 <400> 26
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10 tgaagctgtc gtgcgccgcg tccggcttca cgttctcgaa ctactacatg gcgtgggtgc 180
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15 cgctgtatct gcagatggat tcgctgcgct ccgaggatac cgcgacgtac tactgcgcgc 360
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25 <220>
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VL for vector

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aggcgtogat ctcgtgtcgc tcgtcgcagt cgctggagta ctccgacggc tacacgtatc 180
35 tggagtggta tctgcagaag cccgggcagt cgcgcaggt gctgatctac ggcgtgtcga 240
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<220>
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atcacagatc gtcgacgacc cctcaccatg aatctg 36

55 <210> 29
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<213> Artificial

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 30 <212> DNA
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 Asp Ile Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Thr Pro Gly
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 45 gaa tcc gcg tcg atc tcg tgt cgc tcg tcg cag tcg ctg gag tac tcc 96
 Glu Ser Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Glu Tyr Ser
 20 25 30

 gac ggc tac acg tat ctg gag tgg tat ctg cag aag ccc ggg cag tcg 144
 50 Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
 35 40 45

 ccg cag gtg ctg atc tac ggc gtg tcg aat cgc ttc tcc ggc gtt ccc 192
 Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro
 50 55 60

 55 gat cgc ttc tcc ggc tcc ggc tcc ggg acc gac ttc acg ctg aag atc 240

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	Ser	Arg	Val	Glu	Ala	Glu	Asp	Val	Gly	Val	Tyr	Tyr	Cys	Phe	Gln	Ala	
					85					90					95		
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 gag tcc gcg tgc atc tgc tgt cgc tgc tgc cag tgc ctg gag tac tcc 96
 Glu Ser Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Glu Tyr Ser
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 gag ggc tac acg tat ctg gag tgg tat ctg cag aag ccc ggc cag tgc 144
 Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
 35 40 45
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 ccg cag gtg ctg atc tac ggc gtg tgc aat cgc ttc tcc ggc gtt ccc 192
 Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro
 50 55 60
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 gat cgc ttc tcc ggc tcc ggc tcc ggc acc gac ttc acg ctg aag atc 240
 Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
 65 70 75 80
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 tgc cgc gtc gag gcc gag gac gtc ggc gtg tac tac tgc ttc cag gcg 288
 Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Phe Gln Ala
 85 90 95
 30
 acg cac gat ccg ctg acg ttc ggc cag ggc acg aag ctg gag atc aag 336
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 Glu Ser Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Glu Tyr Ser
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 Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
 35 40 45
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 Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro
 50 55 60
 60
 Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
 65 70 75 80
 65
 Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Phe Gln Ala
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Thr His Asp Pro Leu Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
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gag tcc gcg tcg atc tcg tgt cgc tcg tcg cag tcg ctg gag tac tcc	96
Glu Ser Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Glu Tyr Ser	
20 25 30	
gac ggc tac acg tat ctg gag tgg tat ctg cag aag ccc ggg cag tcg	144
Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser	
35 40 45	
ccg cag gtg ctg atc tac ggc gtg tcg aat cgc ttc tcc ggc gtt ccc	192
Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro	
50 55 60	
gat cgc ttc tcc ggc tcc ggc tcc ggg acc gac ttc acg ctg aag atc	240
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile	
65 70 75 80	
tcg cgc gtc gag gcc gag gac gtc ggc gtg tac tac tgc ttc cag gcg	288
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Phe Gln Ala	
85 90 95	
acg cac gat ccg ctg acg ttc ggg cag ggg acg aag ctg gag atc aag	336
Thr His Asp Pro Leu Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys	
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<400> 37

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Glu Ser Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Glu Tyr Ser	

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10	Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro	50	55	60
15	Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile	65	70	75
20	Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Phe Gln Ala	85	90	95
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55	gag tcc gcg tgc atc tgc tgt cgc tgc tgc cag tgc ctg gag tac tcc			96
	Glu Ser Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Glu Tyr Ser			
	20 25 30			
60	gac ggc tac acg tat ctg gag tgg tat ctg cag aag ccc ggc cag tgc			144
	Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser			
	35 40 45			
65	ccg cag gtg ctg atc tac ggc gtg tgc aat cgc ttc tcc ggc gtt ccc			192
	Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro			
	50 55 60			
70	gat cgc ttc tcc ggc tcc ggc tcc ggg acc gac ttc acg ctg aag atc			240
	Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile			
	65 70 75 80			
75	tcg cgc gtc gaa ccc gag gac gtc ggc gtg tac tac tgc ttc cag gcg			288
	Ser Arg Val Glu Pro Glu Asp Val Gly Val Tyr Tyr Cys Phe Gln Ala			
	85 90 95			
80	acg cac gat ccg ctg acg ttc ggg cag ggg acg aag ctg gag atc aag			336
	Thr His Asp Pro Leu Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys			

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	20 25 30			
20	Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser			
	35 40 45			
	Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro			
25	50 55 60			
	Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile			
	65 70 75 80			
30	Ser Arg Val Glu Pro Glu Asp Val Gly Val Tyr Tyr Cys Phe Gln Ala			
	85 90 95			
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	1 5 10 15			
55	gaa tcc gcg tcg atc tcg tgt cgc tcg tcg cag tcg ctg gag tac tcc			96
	Glu Ser Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Glu Tyr Ser			
	20 25 30			

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	gac ggc tac acg tat ctg gag tgg tat ctg cag aag ccc ggg cag tcg	144
	Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser	
	35 40 45	
5	ccg cag gtg ctg atc tac ggc gtg tcg aat cgc ttc tcc ggc gtt ccc	192
	Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro	
	50 55 60	
10	gat cgc ttc tcc ggc tcc ggc tcc ggg acc gac ttc acg ctg aag atc	240
	Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile	
	65 70 75 80	
15	tcg cgc gtc gag gcc gag gac gtc ggc gtg tac tac tgc ttc cag gcg	288
	Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Phe Gln Ala	
	85 90 95	
20	acg cac gat ccg ctg acg ttc ggg cag ggg acg aag ctg gag atc aag	336
	Thr His Asp Pro Leu Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys	
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45	Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser	
	35 40 45	
50	Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro	
	50 55 60	
55	Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile	
	65 70 75 80	
60	Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Phe Gln Ala	
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1 5 10 15

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gaa tcc gcg tcg atc tcg tgt cgc tcg tcg cag tcg ctg gag tac tcc 96
Glu Ser Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Glu Tyr Ser
20 25 30

20

gac ggc tac acg tat ctg gag tgg tat ctg cag aag ccc ggg cag tcg 144
Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
35 40 45

25

ccg cag gtg ctg atc tac ggc gtg tcg aat cgc ttc tcc ggc gtt ccc 192
Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro
50 55 60

30

gat cgc ttc tcc ggc tcc ggc tcc ggg acc gac ttc acg ctg aag atc 240
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80

35

tcg cgc gtc gaa ccc gag gac gtc ggc gtg tac tac tgc ttc cag gcg 288
Ser Arg Val Glu Pro Glu Asp Val Gly Val Tyr Tyr Cys Phe Gln Ala
85 90 95

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acg cac gat ccg ctg acg ttc ggg cag ggg acg aag ctg gag atc aag 336
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Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
35 40 45

Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro
50 55 60

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Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
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Glu Ser Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Glu Tyr Ser
20 25 30

35 gac ggc tac acg tat ctg gag tgg tat ctg cag aag ccc ggc cag tgc 144
Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
35 40 45

ccg cag gtg ctg atc tac ggc gtg tgc aat cgc ttc tcc ggc gtt ccc 192
Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro
50 55 60

40 gat cgc ttc tcc ggc tcc ggc tcc ggc acc gac ttc acg ctg aag atc 240
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80

45 tgc cgc gtc gag gcc gag gac gtc ggc gtg tac tac tgc ttc cag gcg 288
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Phe Gln Ala
85 90 95

50 acg cac gat ccg ctg acg ttc ggc cag ggc acg aag ctg gag atc aag 336
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35 40 45

15 Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro
50 55 60

20 Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
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Glu Ser Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Glu Tyr Ser
20 25 30

50 gac ggc tac acg tat ctg gag tgg tat ctg cag aag ccc ggg cag tcg 144
Asp Gly Tyr Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
35 40 45

55 ccg cag gtg ctg atc tac ggc gtg tcg aat cgc ttc tcc ggc gtt ccc 192
Pro Gln Val Leu Ile Tyr Gly Val Ser Asn Arg Phe Ser Gly Val Pro
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gat cgc ttc tcc ggc tcc ggc tcc ggg acc gac ttc acg ctg aag atc 240

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5
tcg ctg cgc ctg tcg tgc gcc gcg tcc ggc ttc acg ttc tcg aac tac 96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
20 25 30

10
tac atg gcg tgg gtg cgc cag gcg ccc ggg aag ggg ctg gag tgg gtc 144
Tyr Met Ala Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

15
gcg acg atc tcg tac gac ggc tcg tcg acg tac tat cgc gat tcc gtg 192
Ala Thr Ile Ser Tyr Asp Gly Ser Ser Thr Tyr Tyr Arg Asp Ser Val
50 55 60

20
aag ggg cgc ttc acg atc tcg cgc gac aac gcg aag aac tcg ctg tat 240
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr
65 70 75 80

25
ctg cag atg aac tcg ctg cgc gcc gag gat acc gcc gtg tac tac tgc 288
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

30
gcg cgc cat cgc ggc tac tac tac tcc ggc gcc gcc tac ttc gac tac 336
Ala Arg His Arg Gly Tyr Tyr Tyr Ser Gly Ala Gly Tyr Phe Asp Tyr
100 105 110

35
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Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser
115 120

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Tyr Met Ala Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

55
Ala Thr Ile Ser Tyr Asp Gly Ser Ser Thr Tyr Tyr Arg Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr
65 70 75 80

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Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

5 Ala Arg His Arg Gly Tyr Tyr Tyr Ser Gly Ala Gly Tyr Phe Asp Tyr
100 105 110

10 Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser
115 120

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15 <213> Artificial

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30 tcg ctg cgc ctg tcg tgc gcc gcg tcc ggc ttc acg ttc tcg aac tac 96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
20 25 30

tac atg gcg tgg gtg cgc cag gcg ccc ggg aag ggg ctg gag tgg gtc 144
Tyr Met Ala Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

gcg acg atc tcg tac gac ggc tcg tcg acg tac tat cgc gat tcc gtg 192
Ala Thr Ile Ser Tyr Asp Gly Ser Ser Thr Tyr Arg Asp Ser Val
50 55 60

40 aag ggg cgc ttc acg atc tcg cgc gac aac gcg aag aac tcg ctg tat 240
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr
65 70 75 80

45 ctg cag atg aac tcg ctg cgc gcc gag gat acc gcg acg tac tac tgc 288
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Thr Tyr Tyr Cys
85 90 95

gcg cgc cat cgc ggc tac tac tac tcc ggc gcc ggc tac ttc gac tac 336
Ala Arg His Arg Gly Tyr Tyr Tyr Ser Gly Ala Gly Tyr Phe Asp Tyr
100 105 110

50 tgg ggg cag ggg acg atg gtg acc gtg tcg tcc 369
Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser
115 120

55 <210> 51

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<400> 51

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20 25 30

Tyr Met Ala Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ala Thr Ile Ser Tyr Asp Gly Ser Ser Thr Tyr Tyr Arg Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Thr Tyr Tyr Cys
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Ala Arg His Arg Gly Tyr Tyr Tyr Ser Gly Ala Gly Tyr Phe Asp Tyr
100 105 110

Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser
115 120

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<222> (1) .. (369)

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tcg ctg cgc ctg tcg tgc gcc gcg tcc ggc ttc acg ttc tcg aac tac 96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
20 25 30

tac atg gcg tgg gtg cgc cag gcg ccc ggg aag ggg ctg gag tgg gtc 144
Tyr Met Ala Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val

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	35	40	45	
5	gcg acg atc tcg tac gac ggc tcg tcg acg tac tat cgc gat tcc gtg Ala Thr Ile Ser Tyr Asp Gly Ser Ser Thr Tyr Tyr Arg Asp Ser Val 50 55 60			192
10	aag ggg cgc ttc acg atc tcg cgc gac aac gcg aag aac tcg ctg tat Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr 65 70 75 80			240
15	ctg cag atg aac tcg ctg cgc gcc gag gat acc gcg acg tac tac tgc Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Thr Tyr Tyr Cys 85 90 95			288
20	gcg cgc cat cgc ggc tac tac tac tcc ggc gcc ggc tac ttc gac tac Ala Arg His Arg Gly Tyr Tyr Tyr Ser Gly Ala Gly Tyr Phe Asp Tyr 100 105 110			336
25	tgg ggg cag ggc gtg atg gtg acc gtg tcg tcc Trp Gly Gln Gly Val Met Val Thr Val Ser Ser 115 120			369
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55	Tyr Met Ala Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val 35 40 45			
60	Ala Thr Ile Ser Tyr Asp Gly Ser Ser Thr Tyr Tyr Arg Asp Ser Val 50 55 60			
65	Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr 65 70 75 80			
70	Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Thr Tyr Tyr Cys 85 90 95			
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      tcg atg cgc ctg tcg tgc gcc gcg tcc ggc ttc acg ttc tcg aac tac      96
20     Ser Met Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
      20          25          30

      tac atg gcg tgg gtg cgc cag gcg ccc ggg aag ggg ctg gag tgg gtc      144
25     Tyr Met Ala Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
      35          40          45

      gcg acg atc tcg tac gac ggc tcg tcg acg tac tat cgc gat tcc gtg      192
      Ala Thr Ile Ser Tyr Asp Gly Ser Ser Thr Tyr Tyr Arg Asp Ser Val
      50          55          60

30     aag ggg cgc ttc acg atc tcg cgc gac aac gcg aag aac tcg ctg tat      240
      Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr
      65          70          75          80

      ctg cag atg aac tcg ctg cgc gcc gag gat acc gcc gtg tac tac tgc      288
35     Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
      85          90          95

      gcg cgc cat cgc ggc tac tac tac tcc ggc gcc ggc tac ttc gac tac      336
      Ala Arg His Arg Gly Tyr Tyr Tyr Ser Gly Ala Gly Tyr Phe Asp Tyr
      100         105         110

40     tgg ggg cag ggc gtg atg gtg acc gtg tcg tcc      369
      Trp Gly Gln Gly Val Met Val Thr Val Ser Ser
      115          120

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50     <220>
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      <400>  55

55     Glu Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
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	Ser	Met	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe	Ser	Asn	Tyr	
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45	tac	atg	gcg	tgg	gtg	cgc	cag	gcg	ccc	ggg	aag	ggg	ctg	gag	tgg	gtc	144
	Tyr	Met	Ala	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Val	
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50	Ala	Thr	Ile	Ser	Tyr	Asp	Gly	Ser	Ser	Thr	Tyr	Tyr	Arg	Asp	Ser	Val	
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	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn	Ser	Leu	Tyr	
55	65					70					75					80	
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	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Thr	Tyr	Tyr	Cys	
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	Ala	Arg	His	Arg	Gly	Tyr	Tyr	Tyr	Ser	Gly	Ala	Gly	Tyr	Phe	Asp	Tyr	
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	Tyr	Met	Ala	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Val	
30			35					40					45				
	Ala	Thr	Ile	Ser	Tyr	Asp	Gly	Ser	Ser	Thr	Tyr	Tyr	Arg	Asp	Ser	Val	
		50					55					60					
35	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn	Ser	Leu	Tyr	
	65					70					75					80	
40	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Thr	Tyr	Tyr	Cys	
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	Ala	Arg	His	Arg	Gly	Tyr	Tyr	Tyr	Ser	Gly	Ala	Gly	Tyr	Phe	Asp	Tyr	
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10 tcg atg cgc ctg tcg tgc gcc gcg tcc ggc ttc acg ttc tcg aac tac 96
 Ser Met Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
 20 25 30

15 tac atg gcg tgg gtg cgc cag gcg ccc ggg aag ggg ctg gag tgg gtc 144
 Tyr Met Ala Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35 40 45

20 gcg acg atc tcg tac gac ggc tcg tcg acg tac tat cgc gat tcc gtg 192
 Ala Thr Ile Ser Tyr Asp Gly Ser Ser Thr Tyr Tyr Arg Asp Ser Val
 50 55 60

25 aag ggg cgc ttc acg atc tcg cgc gac aac gcg aag tcg tcg ctg tat 240
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Ser Ser Leu Tyr
 65 70 75 80

25 ctg cag atg aac tcg ctg cgc gcc gag gat acc gcg acg tac tac tgc 288
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Thr Tyr Tyr Cys
 85 90 95

30 gcg cgc cat cgc ggc tac tac tac tcc ggc gcc ggc tac ttc gac tac 336
 Ala Arg His Arg Gly Tyr Tyr Tyr Ser Gly Ala Gly Tyr Phe Asp Tyr
 100 105 110

30 tgg ggg cag ggc gtg atg gtg acc gtg tcg tcc 369
 Trp Gly Gln Gly Val Met Val Thr Val Ser Ser
 115 120

35 <210> 59
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40 <220>
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 20 25 30

50 Tyr Met Ala Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35 40 45

55 Ala Thr Ile Ser Tyr Asp Gly Ser Ser Thr Tyr Tyr Arg Asp Ser Val
 50 55 60

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Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Ser Ser Leu Tyr
 65 70 75 80

5 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Thr Tyr Tyr Cys
 85 90 95

10 Ala Arg His Arg Gly Tyr Tyr Tyr Ser Gly Ala Gly Tyr Phe Asp Tyr
 100 105 110

15 Trp Gly Gln Gly Val Met Val Thr Val Ser Ser
 115 120

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 <212> DNA
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55 ctgctcagcg ccatacagct ggaccgctgc ctgcaggtgg tgtggccggg gtgggcgcag 420

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 35 40 45

40 Glu Asn Gly Val Ile Leu Phe Val Val Gly Cys Arg Met Arg Gln Thr
 50 55 60

45 Val Val Thr Thr Trp Val Leu His Leu Ala Leu Ser Asp Leu Leu Ala
 65 70 75 80

50 Ser Ala Ser Leu Pro Phe Phe Thr Tyr Phe Leu Ala Val Gly His Ser
 85 90 95

Trp Glu Leu Gly Thr Thr Phe Cys Lys Leu His Ser Ser Ile Phe Phe
 100 105 110

55 Leu Asn Met Phe Ala Ser Gly Phe Leu Leu Ser Ala Ile Ser Leu Asp
 115 120 125

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	Asp	Gly	Arg	Ile	Met	Cys	Tyr	Tyr	Asn	Val	Leu	Leu	Leu	Asn	Pro	Gly	
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 <213> Artificial

10 <220>
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15 <210> 65
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 <212> DNA
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20 <220>
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25

Claims

- 30 1. An antibody or the antibody fragment thereof which recognizes at least one of 192th glycine and 194th aspartic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2 and binds thereto.
- 35 2. The antibody or the antibody fragment thereof according to claim 1, wherein the antibody recognizes at least one amino acid residue selected from the group consisting of 12th proline, 13th isoleucine, 14th leucine, 15th glutamic acid, 177th aspartic acid, 178th glycine, 179th arginine, 180th isoleucine, 181th methionine, 182nd cysteine, 183rd tyrosine, 184th tyrosine, 185th asparagine, 186th valine, 187th leucine, 188th leucine, 189th leucine, 195th arginine, 196th aspartic acid, 197th alanine, and 198th threonine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2 and binds thereto.
- 40 3. The antibody or the antibody fragment thereof according to claim 1 or 2, wherein the antibody recognizes at least one amino acid residue selected from the group consisting of the following (a) to (g):
- (a) 12th proline, 14th leucine, and 15th glutamic acid in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2;
- 45 (b) 177th aspartic acid, 178th glycine, and 179th arginine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2;
- (c) 180th isoleucine and 181st methionine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2;
- 50 (d): 183rd tyrosine, 184th tyrosine, and 185th asparagine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2;
- (e) 187th leucine, 188th leucine, and 189th leucine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2;
- (f) 195th arginine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2; and
- 55 (g) 196th aspartic acid and 198th threonine in the amino acid sequence of human CRTH2 represented by SEQ ID NO: 2.
4. The antibody or the antibody fragment thereof according to any one of claims 1 to 3, wherein the antibody is any one selected from the group consisting of the following (a) to (d):

(a) an antibody in which complementarity determining regions (hereinafter, abbreviated as CDR) 1 to 3 of an antibody heavy chain variable region (hereinafter, abbreviated as VH) comprise amino acid sequences represented by SEQ ID NOS: 20 to 22, respectively, and CDRs 1 to 3 of an antibody light chain variable regions (hereinafter, abbreviated as VL) comprise amino acid sequences represented by SEQ ID NOS: 23 to 25, respectively;

(b) an antibody which comprises VH comprising the amino acid sequence represented by SEQ ID NO: 49 or the amino acid sequence obtained by introducing at least one modification selected from modifications of substituting 18th leucine with methionine, 77th asparagine with serine, 93th valine with threonine, and 117th threonine with valine in the amino acid sequence represented by SEQ ID NO: 49 and VL comprising the amino acid sequence represented by SEQ ID NO: 33 or the amino acid sequence obtained by at least one modification selected from modifications of substituting 2nd isoleucine with valine, 4th methionine with leucine, 15th proline with leucine, and 85th alanine with proline in the amino acid sequence represented by SEQ ID NO: 33;

(c) an antibody which comprises VH comprising any one of the amino acid sequences represented by SEQ ID NOS: 49, 51, 53, 55, 57, and 59 and VL comprising any one of the amino acid sequences represented by SEQ ID NOS: 33, 35, 37, 39, 41, 43, 45, and 47; and

(d) an antibody which comprises VH comprising the amino acid sequence represented by SEQ ID NO: 17 and VL comprising the amino acid sequence represented by SEQ ID NO: 19.

5. The antibody or the antibody fragment thereof according to any one of claims 1 to 4, wherein the antibody has at least one characteristic selected from the group consisting of the following (a) to (h):

(a) reactivity of the antibody against human CRTH2 does not decrease in the presence of a ligand of human CRTH2;

(b) neutralizing activity is not shown by the antibody;

(c) antibody-dependent cell-mediated cytotoxicity (ADCC) is shown by the antibody;

(d) a reaction of the antibody with at least one of mast cells and Th1 cells does not occur;

(e) a reaction of the antibody with at least one selected from eosinophils, basophils, Th2 cells, and type 2 innate lymphoid cells (ILC2) occurs;

(f) agonist activity is not shown by the antibody;

(g) a signal caused by the ligand of human CRTH2 is not enhanced by the antibody; and

(h) reactivity of the antibody against human CRTH2 in an activated or inactivated state does not change.

6. The antibody or the antibody fragment thereof according to any one of claims 1 to 5, wherein the antibody comprises a human Fc region.

7. The antibody or the antibody fragment thereof according to any one of claims 1 to 6, wherein the antibody is a monoclonal antibody.

8. The antibody or the antibody fragment thereof according to any one of claims 1 to 7, wherein the antibody is a recombinant antibody.

9. The recombinant antibody or the antibody fragment thereof according to claim 8, wherein the recombinant antibody is any one recombinant antibody selected from a human chimeric antibody, a human CDR-grafted antibody, and a human antibody.

10. The antibody or the antibody fragment thereof according to any one of claims 1 to 9, wherein the antibody is an antibody which binds to monkey CRTH2.

11. The antibody fragment according to any one of claims 1 to 10, wherein the antibody fragment is any one selected from Fab, Fab', F(ab')₂, scFv, diabody, dsFv, and peptide including CDR.

12. A hybridoma which generates the antibody or the antibody fragment thereof according to any one of claims 1 to 11.

13. A DNA which encodes the antibody or the antibody fragment thereof according to any one of claims 1 to 11.

14. A recombinant vector which comprises the DNA according to claim 13.

15. A transformant which is obtained by introducing the recombinant vector according to claim 14 into a host cell.

16. A method of producing the antibody or the antibody fragment thereof according to any one of claims 1 to 11, comprising:

culturing the hybridomas according to claim 12 or the transformant according to claim 15 in a culture medium;
producing and accumulating the antibody or the antibody fragment thereof according to any one of claims 1 to 11 in a culture; and
collecting the antibody or the antibody fragment thereof from the culture.

17. A therapeutic agent for a disease associated with human CRTH2, comprising:
the antibody or the antibody fragment thereof according to any one of claims 1 to 11 as an active ingredient.

18. A diagnostic agent for a disease associated with human CRTH2, comprising:
the antibody or the antibody fragment thereof according to any one of claims 1 to 11 as an active ingredient.

19. The agent according to claim 17 or 18, wherein the disease associated with CRTH2 is an allergic disease; an autoimmune disease; a disease accompanied by at least one of increase and hyperergasia of eosinophils; a disease accompanied by at least one of increase and hyperergasia of Th2 cells; or a disease accompanied by at least one of increase and hyperergasia of type 2 innate lymphoid cells (ILC2).

20. A method of treating a disease associated with human CRTH2 comprising:
administrating an effective amount of the antibody or the antibody fragment thereof according to any one of claims 1 to 11.

21. A method of diagnosing a disease associated with human CRTH2 comprising:
administrating an effective amount of the antibody or the antibody fragment thereof according to any one of claims 1 to 11.

22. The method according to claim 20 or 21, wherein the disease associated with human CRTH2 is an allergic disease; an autoimmune disease; a disease accompanied by at least one of increase and hyperergasia of eosinophils; a disease accompanied by at least one of increase and hyperergasia of Th2 cells; or a disease accompanied by at least one of increase and hyperergasia of ILC2.

23. The antibody or the antibody fragment thereof according to any one of claims 1 to 11, for a use in at least one of treatment and diagnosis for a disease associated with human CRTH2.

24. The antibody or the antibody fragment thereof according to claim 23, wherein the disease associated with human CRTH2 is a an allergic disease; an autoimmune disease; a disease accompanied by at least one of increase and hyperergasia of eosinophils; a disease accompanied by at least one of increase and hyperergasia of Th2 cells; or a disease accompanied by at least one of increase and hyperergasia of ILC2.

25. Use of the antibody or the antibody fragment thereof according to any one of claims 1 to 11, for the manufacture of at least one of a therapeutic agent and a diagnostic agent for a disease associated with human CRTH2.

26. The use according to claim 25, wherein the disease associated with human CRTH2 is an allergic disease; an autoimmune disease; a disease accompanied by at least one of increase and hyperergasia of eosinophils; a disease accompanied by at least one of increase and hyperergasia of Th2 cells; or a disease accompanied by at least one of increase and hyperergasia of ILC2.

[Fig. 1]

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Lym2 VL	DVVLTQTPVSLSVTLGDQASISC	RSSQSLEYS DG YTYLE		WYLQKPGQSPQVLIY	GVS NRFS
LV0	DIVMTQTPLSLPVT PGESASISC			WYLQKPGQSPQVLIY	
LV1	DIVMTQTPLSLPVT LGESASISC			WYLQKPGQSPQVLIY	
LV2a	DIVLTQTPLSLPVT LGESASISC			WYLQKPGQSPQVLIY	
LV2b	DIVMTQTPLSLPVT LGESASISC			WYLQKPGQSPQVLIY	
LV2c	DVVLTQTPVSLPVT PGESASISC	CDR L1		WYLQKPGQSPQVLIY	CDR L2
LV3a	DVVLTQTPVSLPVT PGESASISC			WYLQKPGQSPQVLIY	
LV3b	DVVLTQTPVSLPVT LGESASISC			WYLQKPGQSPQVLIY	
LV4	DVVLTQTPVSLPVT LGESASISC			WYLQKPGQSPQVLIY	

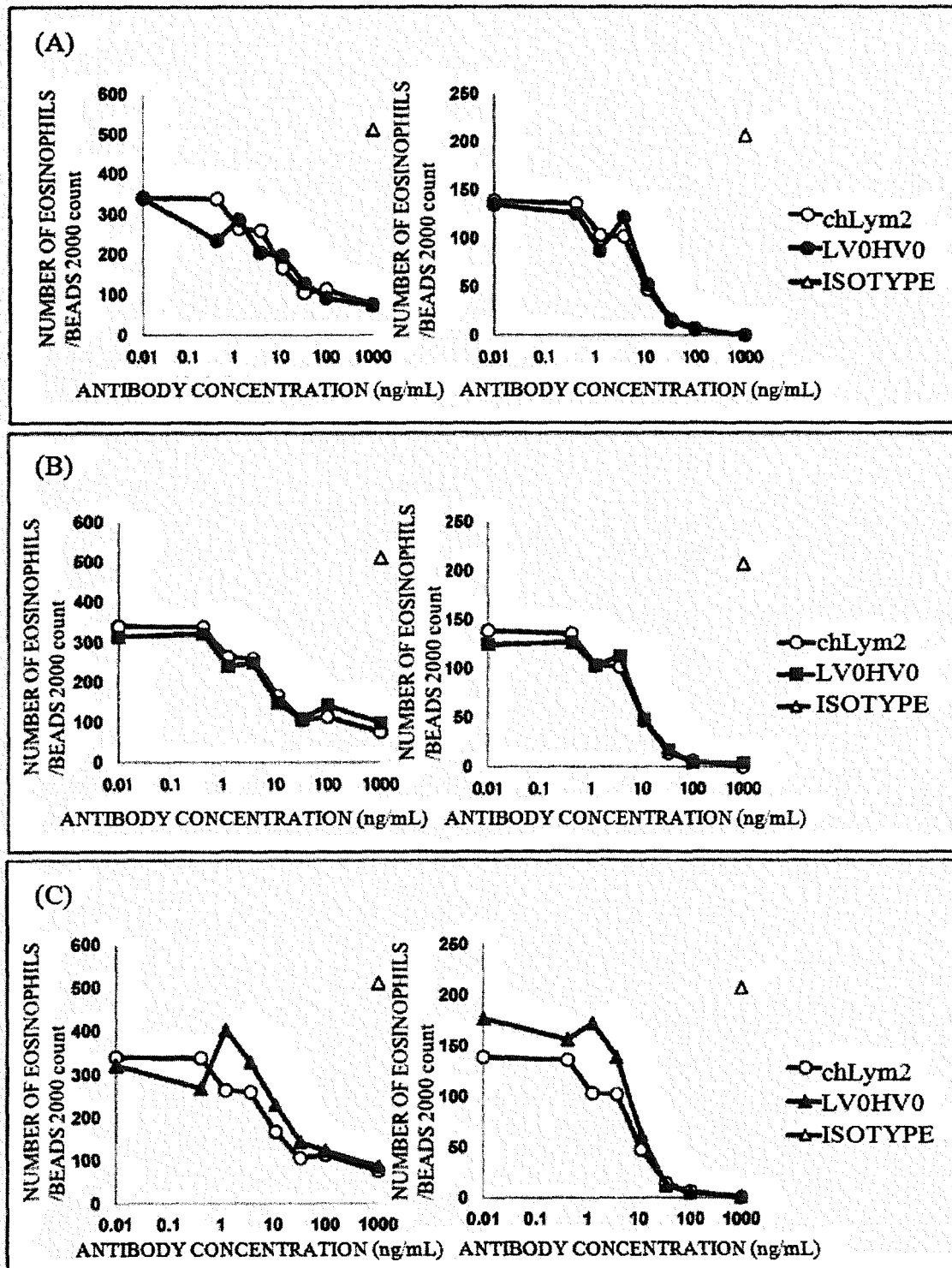
		23456789012345678901234567890123	456789012	3456789012
Lym2 VL	GVPDRFSGSGSGTDFTLKISRVEPEDLG VYYC	FQATHDPLT		FGSGTKLEIK
LV0	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC			FGQGTKLEIK
LV1	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC			FGQGTKLEIK
LV2a	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC			FGQGTKLEIK
LV2b	GVPDRFSGSGSGTDFTLKISRVEPEDVGVYYC			FGQGTKLEIK
LV2c	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	CDR L3		FGQGTKLEIK
LV3a	GVPDRFSGSGSGTDFTLKISRVEPEDVGVYYC			FGQGTKLEIK
LV3b	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC			FGQGTKLEIK
LV4	GVPDRFSGSGSGTDFTLKISRVEPEDVGVYYC			FGQGTKLEIK

[Fig.2]

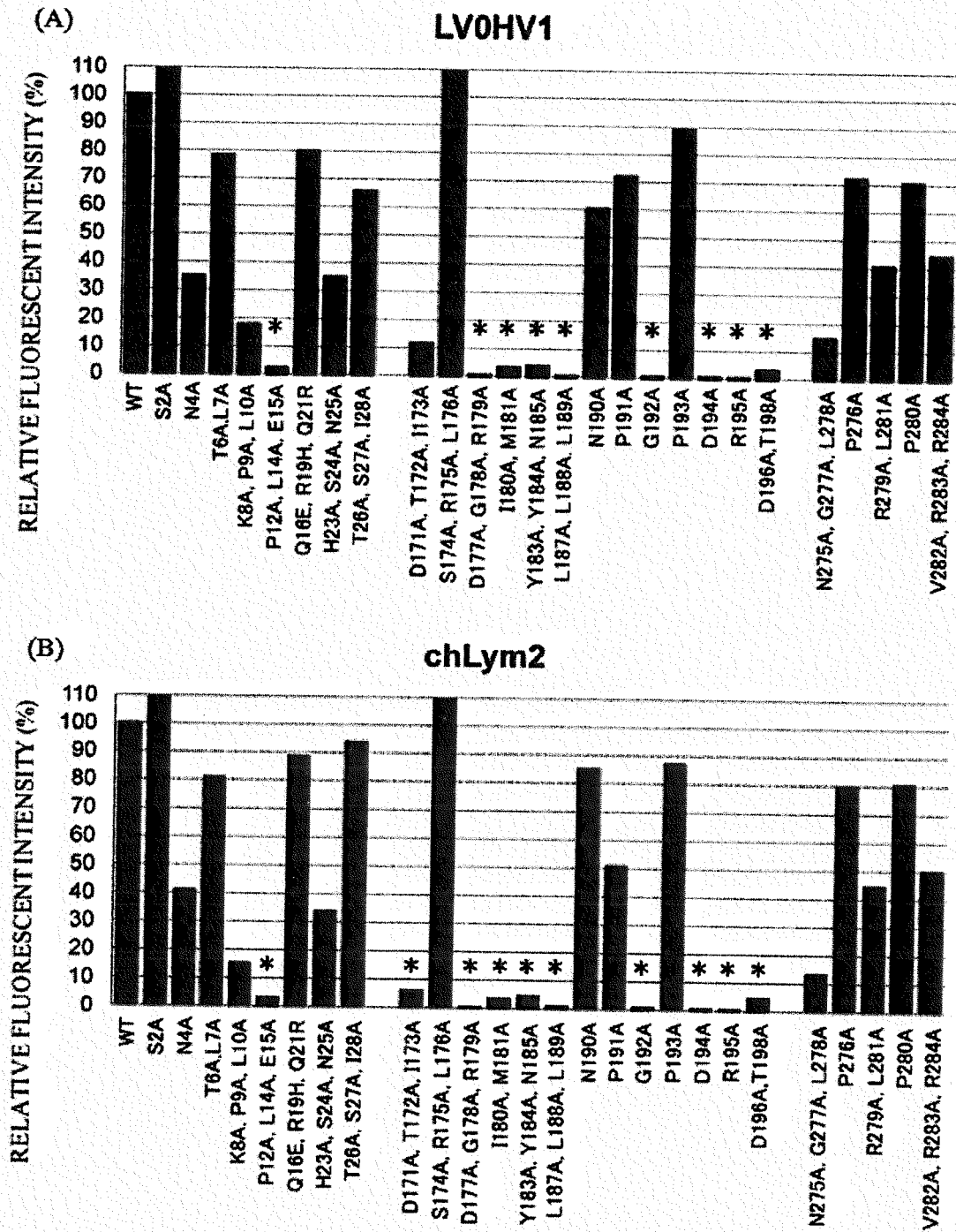
		123456789012345678901234567890	12345	67890123456789	01234567890123456
Lym2 VH	EVQLVESGGGLVQPGRSMKLSCAASGFTFS	NYMA		WVRQAPKGLEWVA	TISYDGSSTYYRDSVKG
HV0	EVQLVESGGGVVQPGRSLRLSCAASGFTFS			WVRQAPKGLEWVA	
HV1	EVQLVESGGGVVQPGRSLRLSCAASGFTFS			WVRQAPKGLEWVA	
HV2a	EVQLVESGGGVVQPGRSLRLSCAASGFTFS			WVRQAPKGLEWVA	
HV2b	EVQLVESGGGVVQPGRSMRLSCAASGFTFS	CDR H1		WVRQAPKGLEWVA	CDR H2
HV3	EVQLVESGGGVVQPGRSMRLSCAASGFTFS			WVRQAPKGLEWVA	
HV4	EVQLVESGGGVVQPGRSMRLSCAASGFTFS			WVRQAPKGLEWVA	

		78901234567890123456789012345678	90123456789012	34567890123
Lym2 VH	RFTISRDNASTLYLQMNSLRSED TATYYCAR	HRGYYYSGAGYFDY		WGQGMVTVSS
HV0	RFTISRDNAKNSLYLQMNSLRAED TAVYYCAR			WGQGMVTVSS
HV1	RFTISRDNAKNSLYLQMNSLRAED TATYYCAR			WGQGMVTVSS
HV2a	RFTISRDNAKNSLYLQMNSLRAED TATYYCAR			WGQGMVTVSS
HV2b	RFTISRDNAKNSLYLQMNSLRAED TAVYYCAR	CDR H3		WGQGMVTVSS
HV3	RFTISRDNAKNSLYLQMNSLRAED TATYYCAR			WGQGMVTVSS
HV4	RFTISRDNAKSSLYLQMNSLRAED TATYYCAR			WGQGMVTVSS

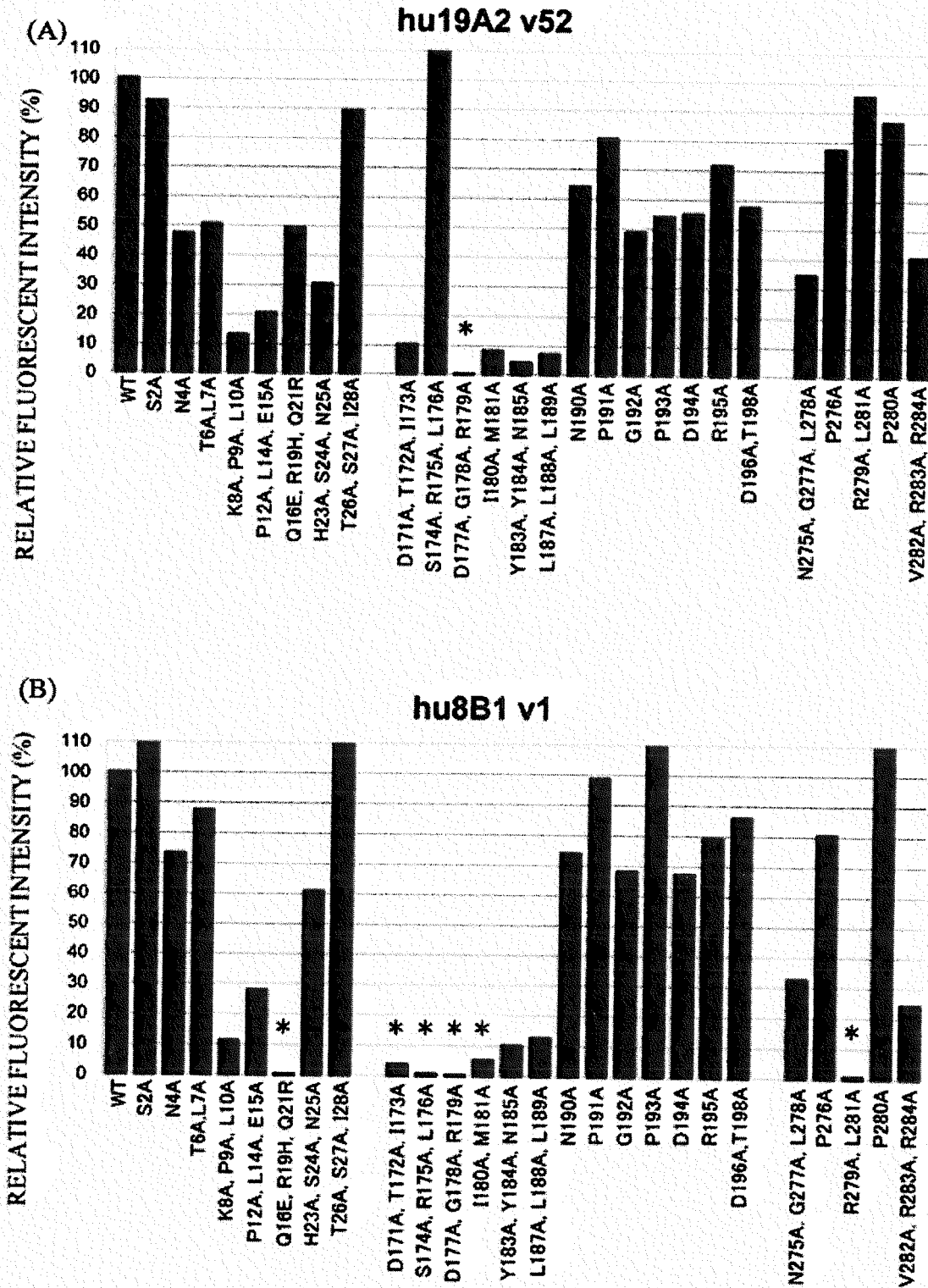
[FIG. 3]



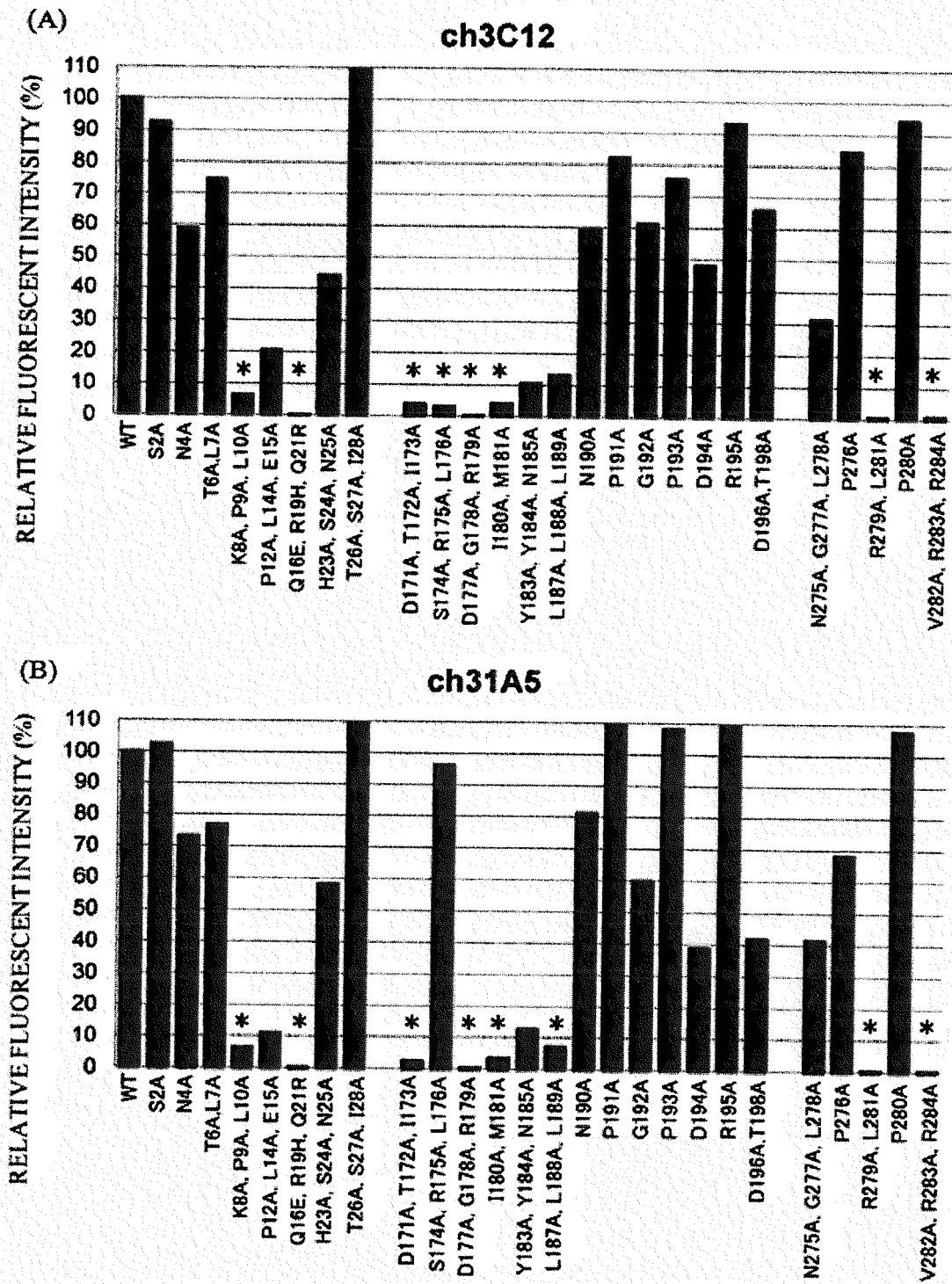
[FIG. 4]



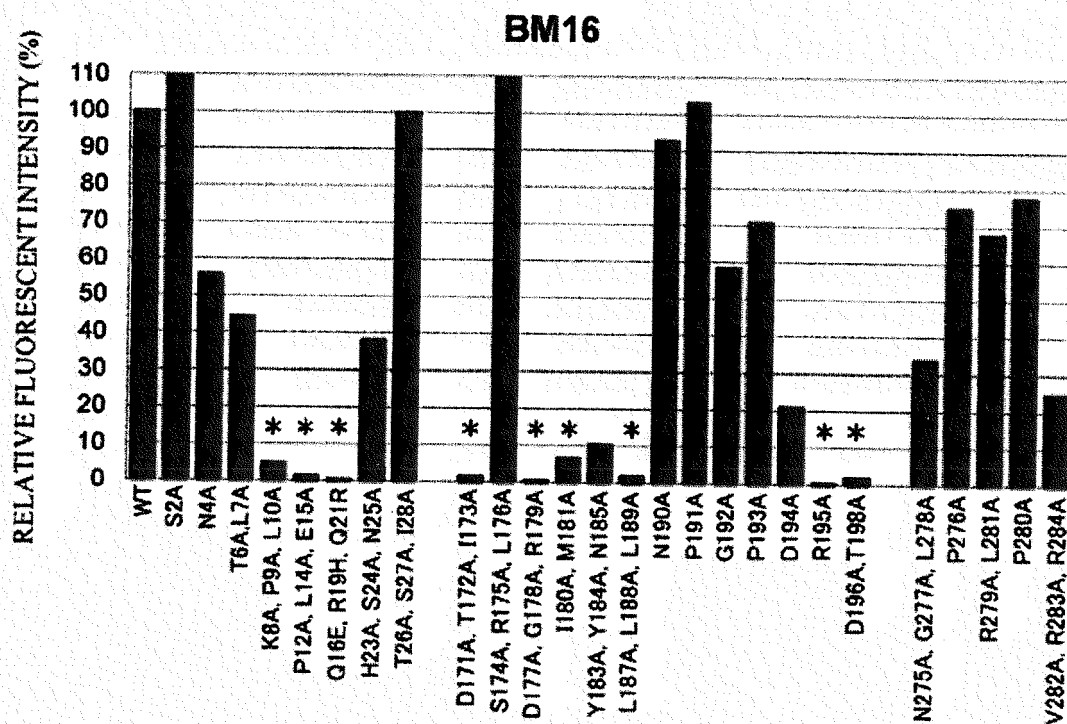
[FIG. 5]



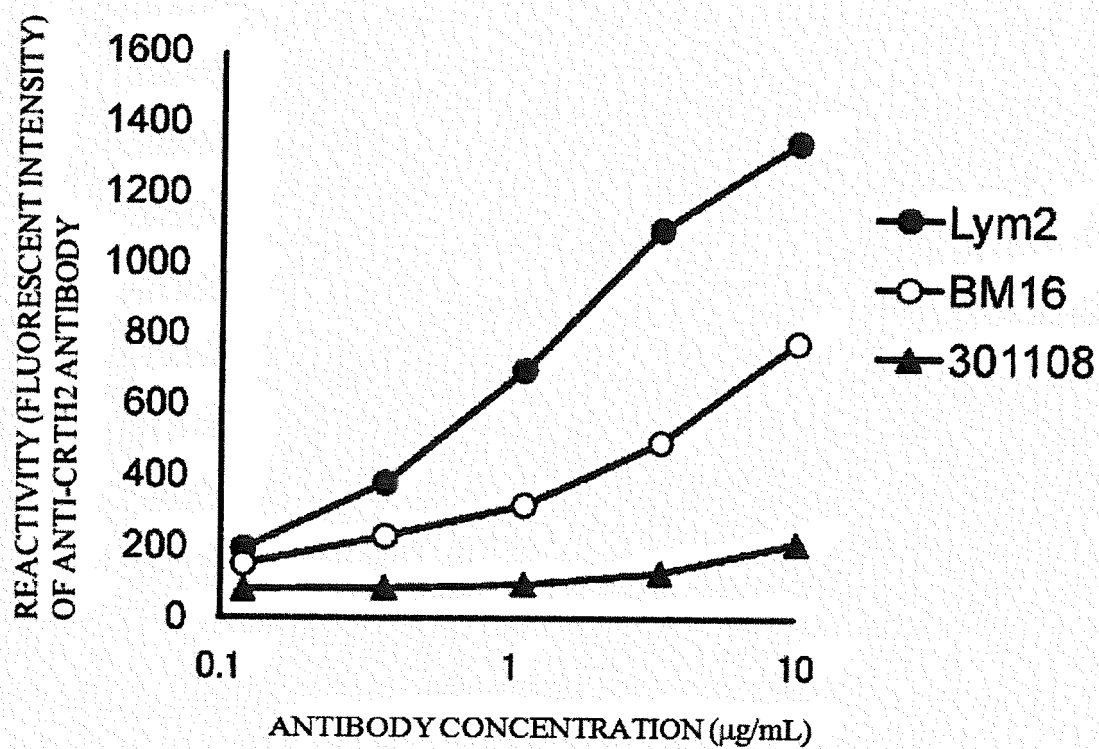
[FIG. 6]



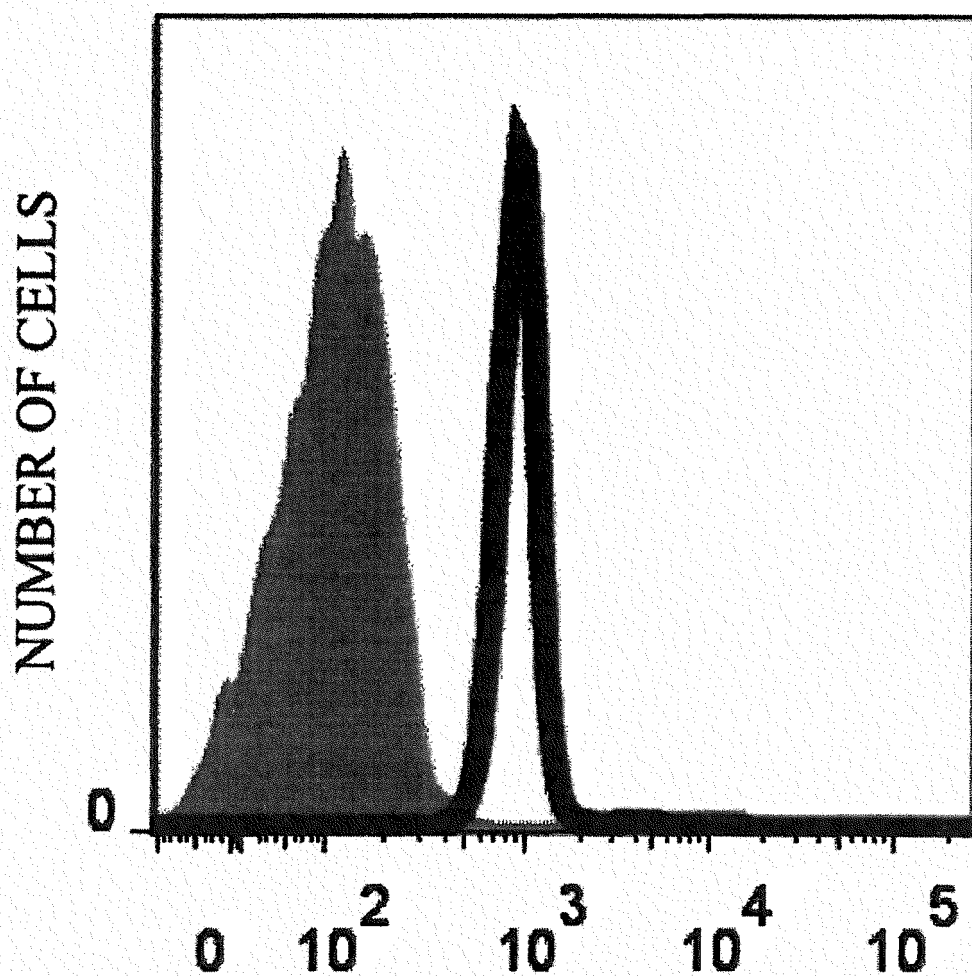
[FIG. 7]



[FIG. 8]

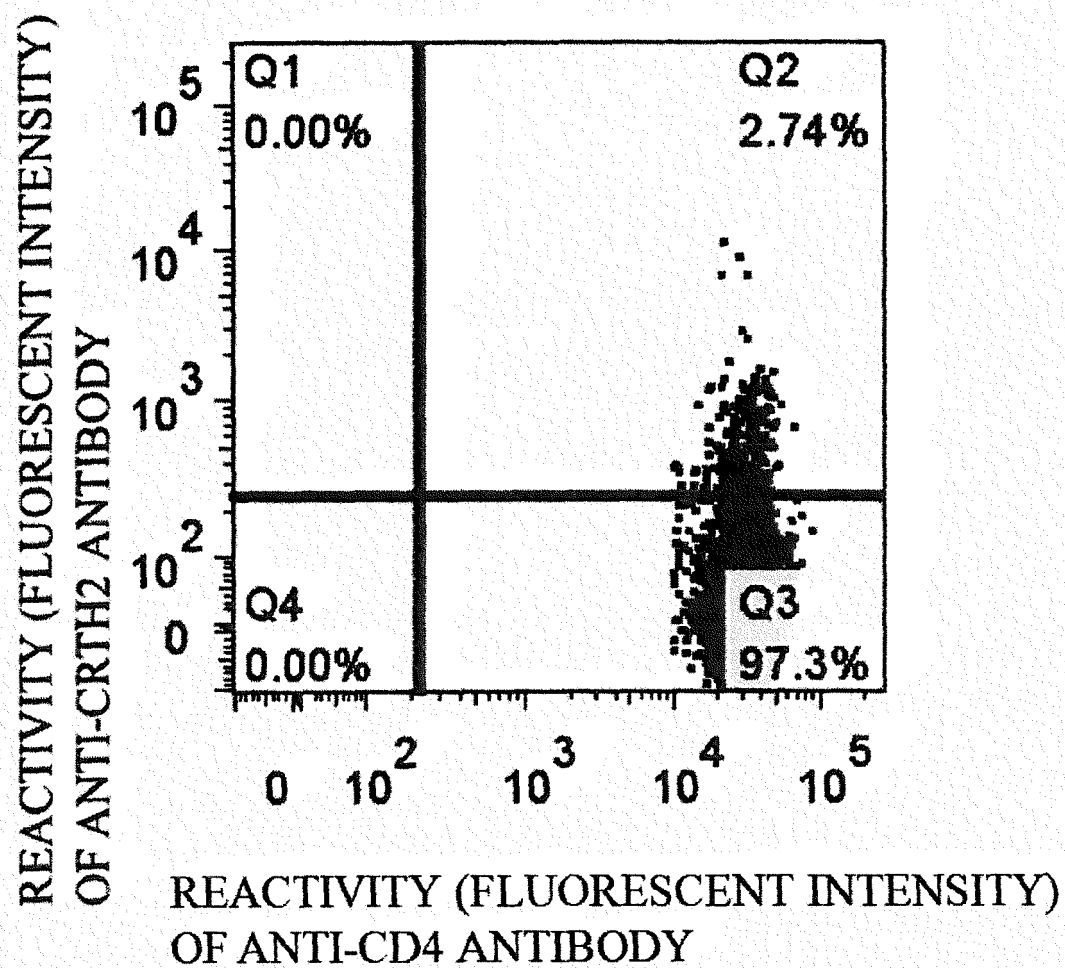


[FIG. 9]



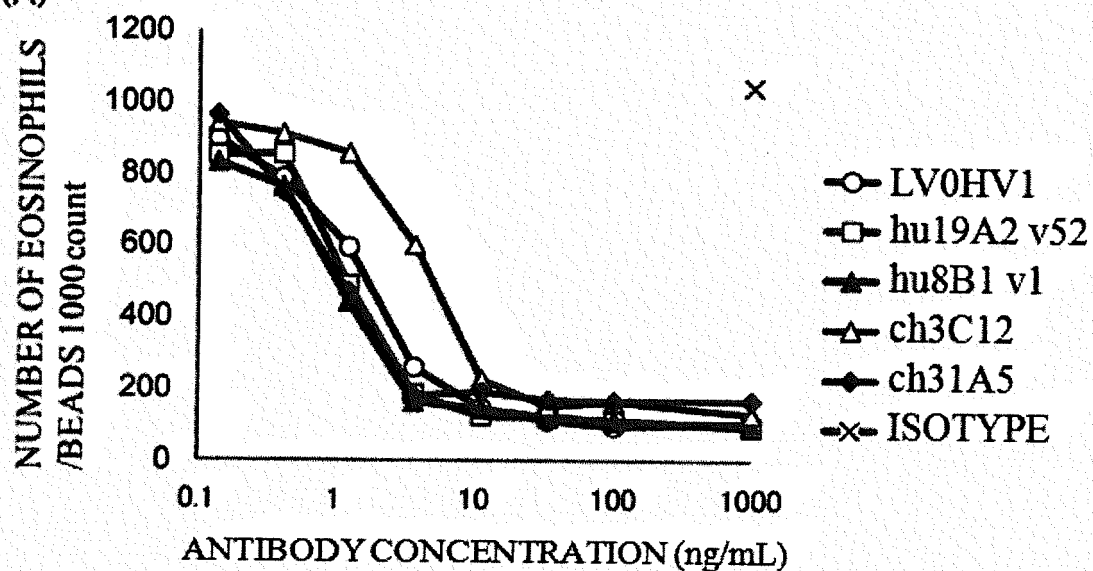
REACTIVITY (FLUORESCENT INTENSITY)
OF ANTI-CRTH2 ANTIBODY

[FIG. 10]

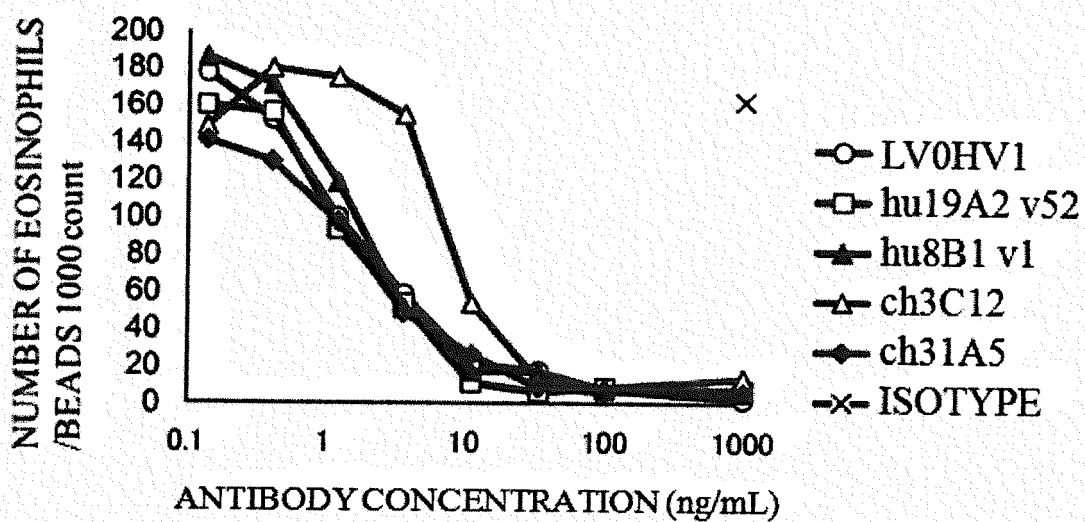


[FIG. 11]

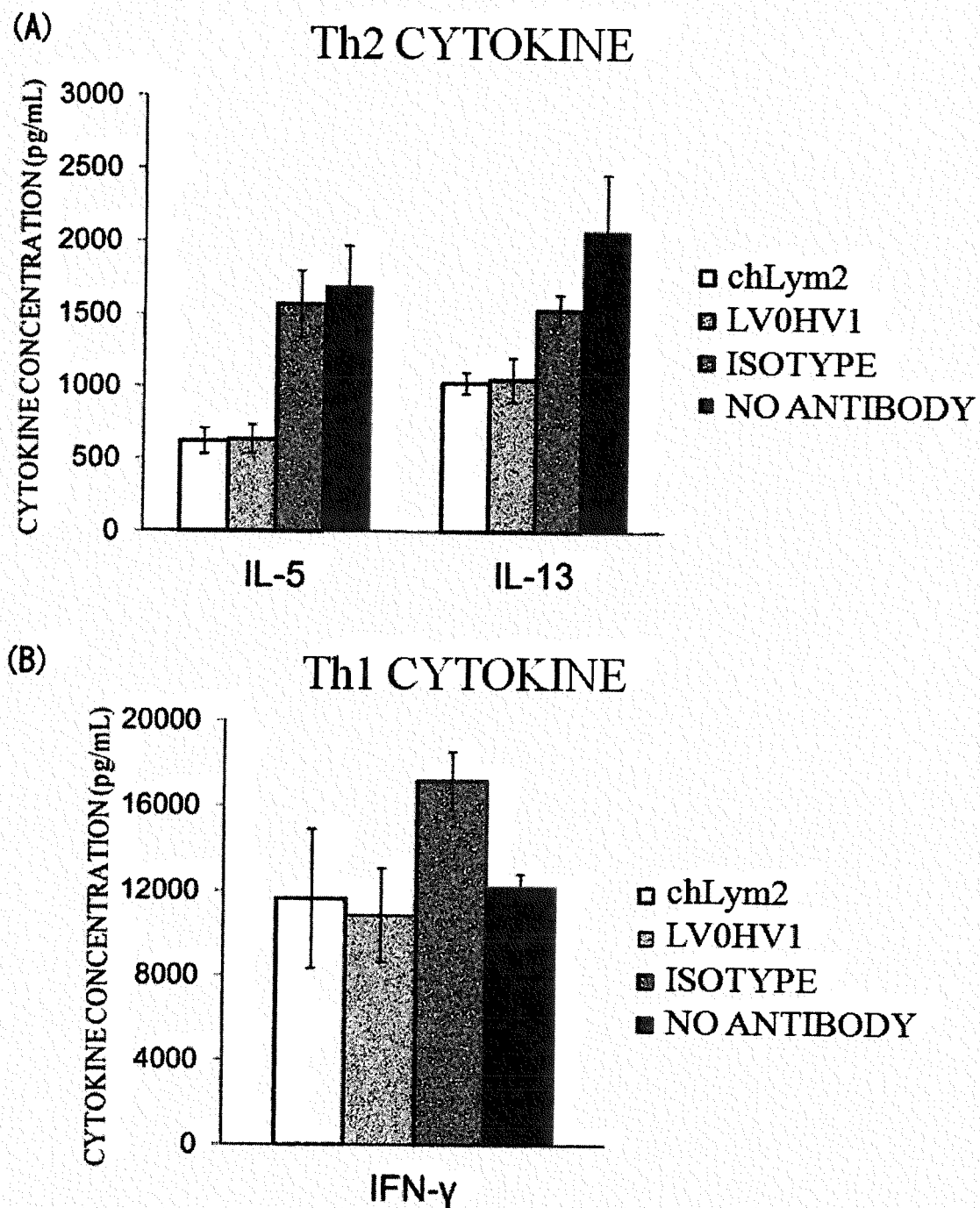
(A)



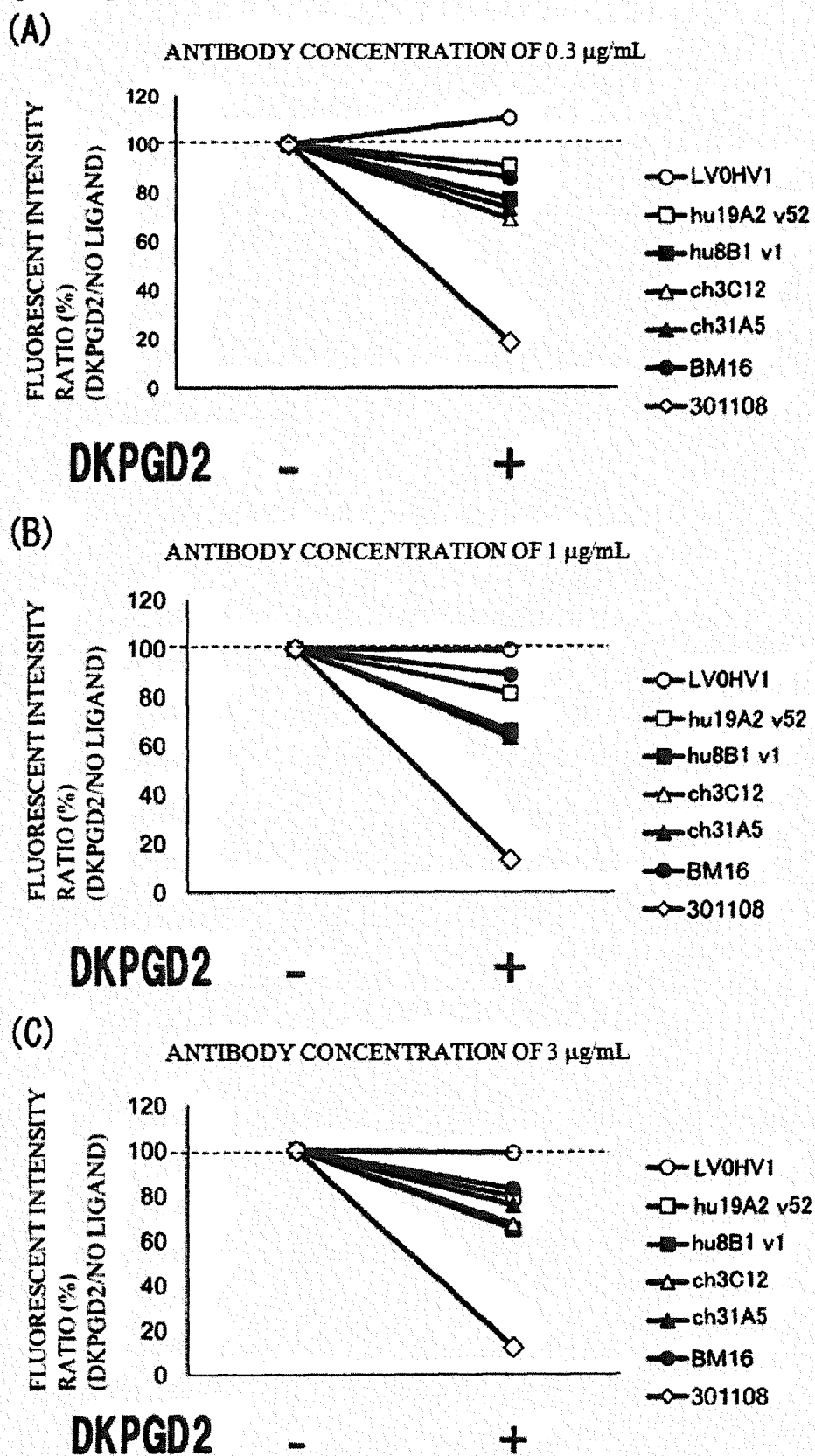
(B)



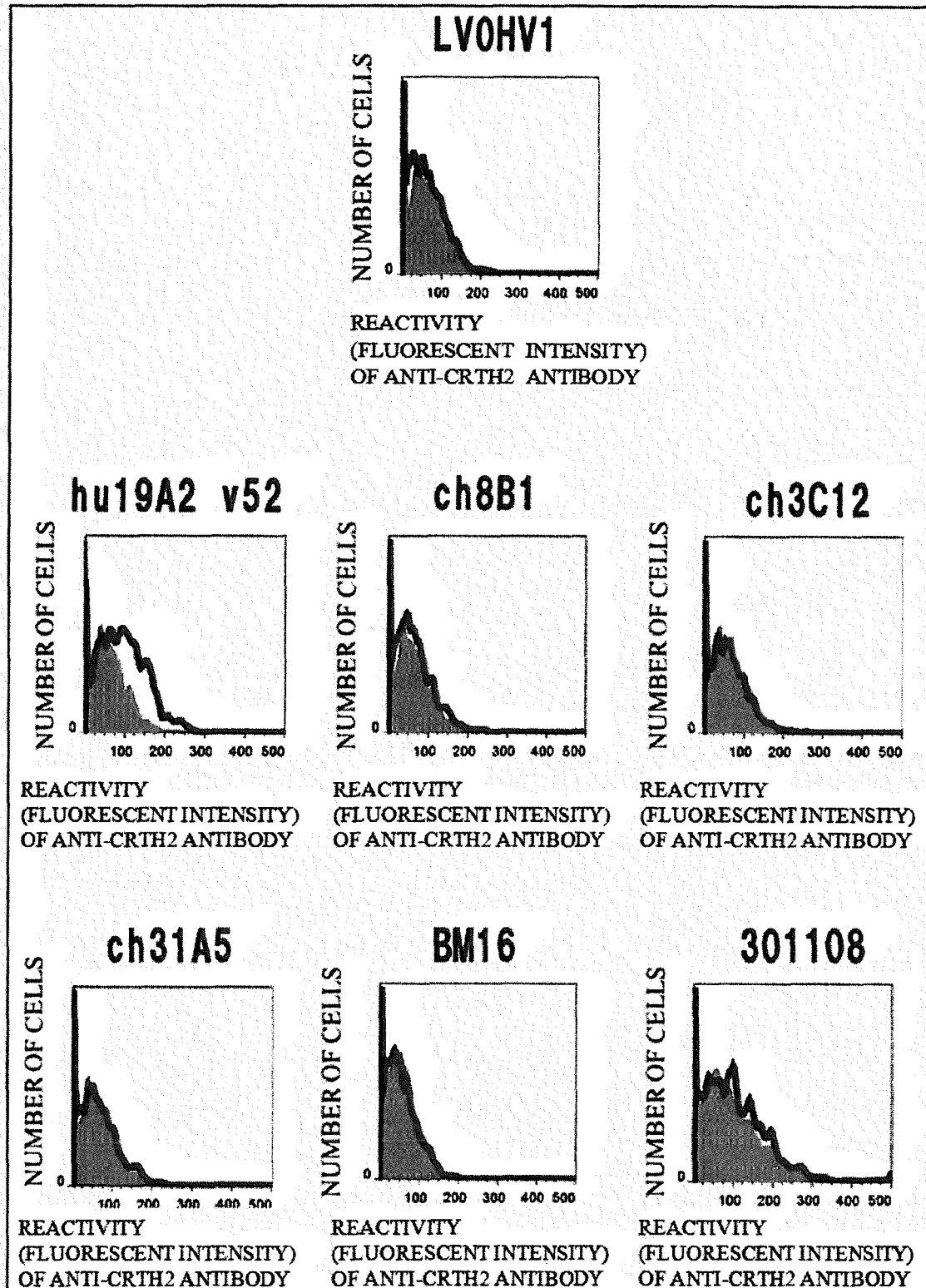
[FIG. 12]



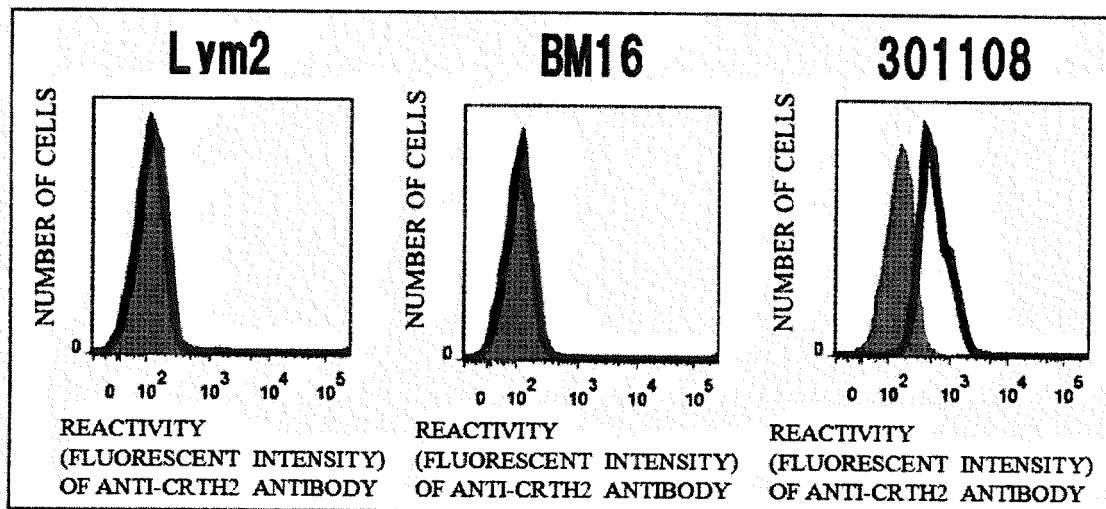
[FIG. 13]



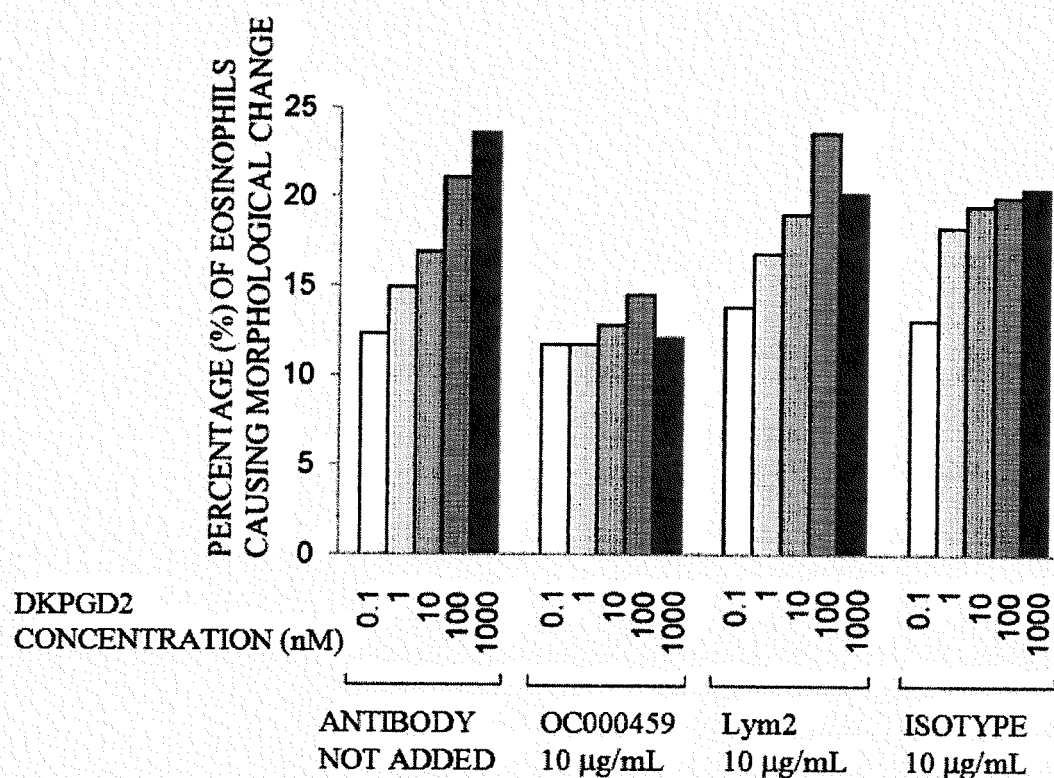
[FIG. 14]



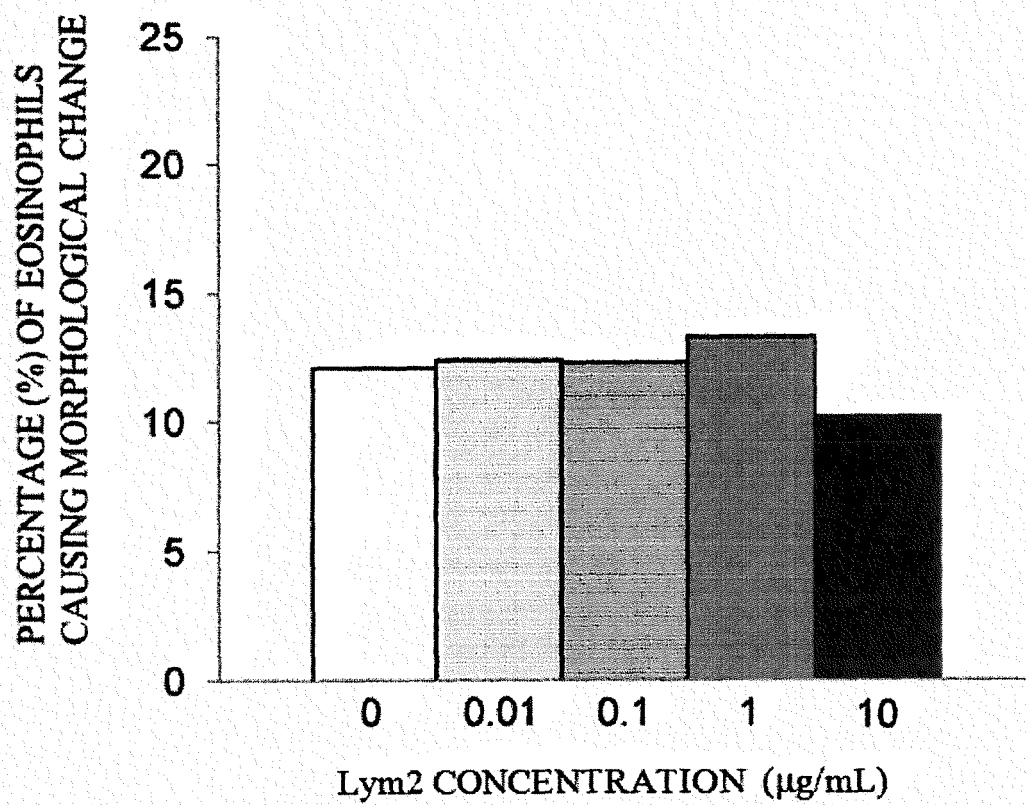
[FIG. 15]



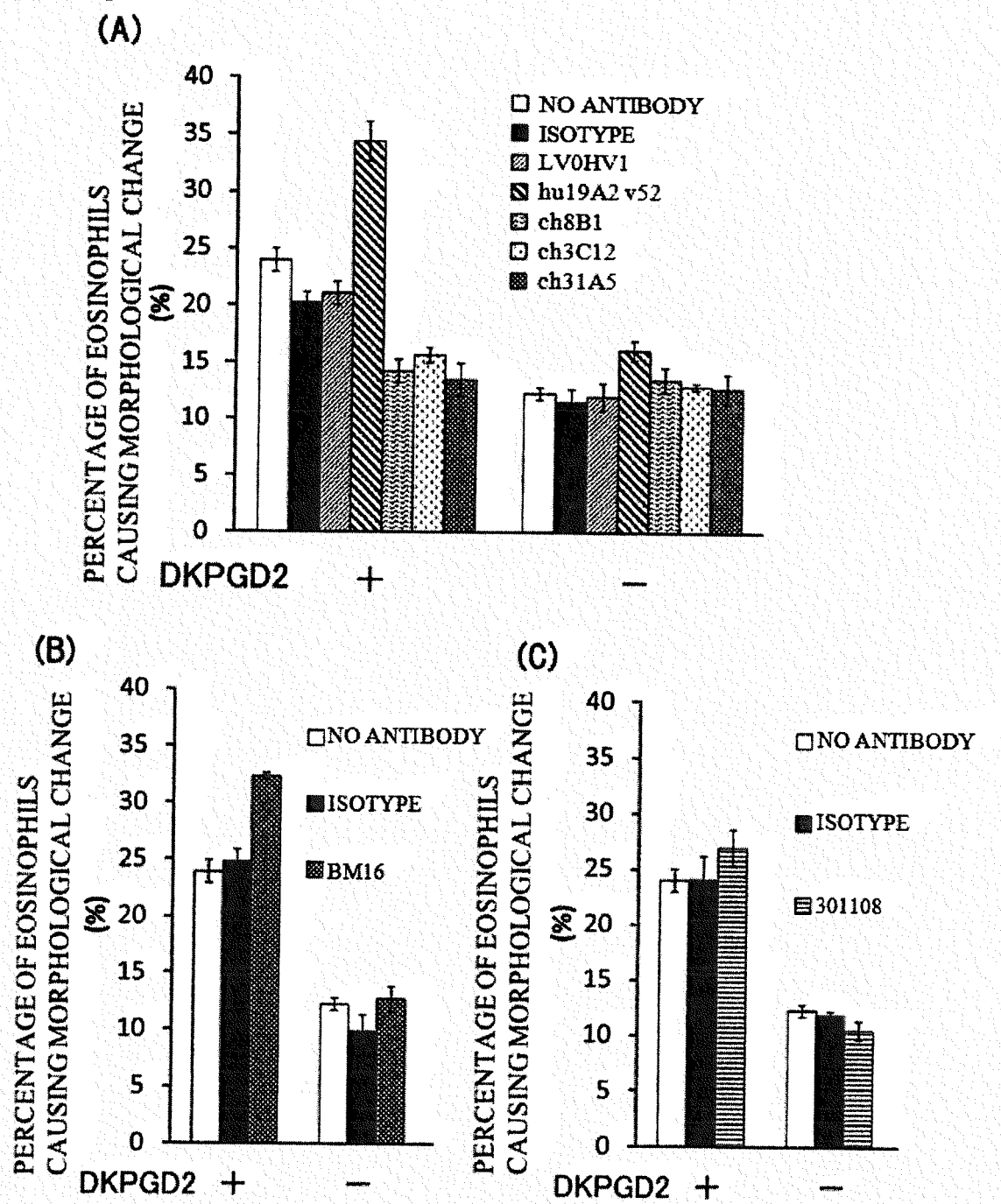
[FIG. 16]



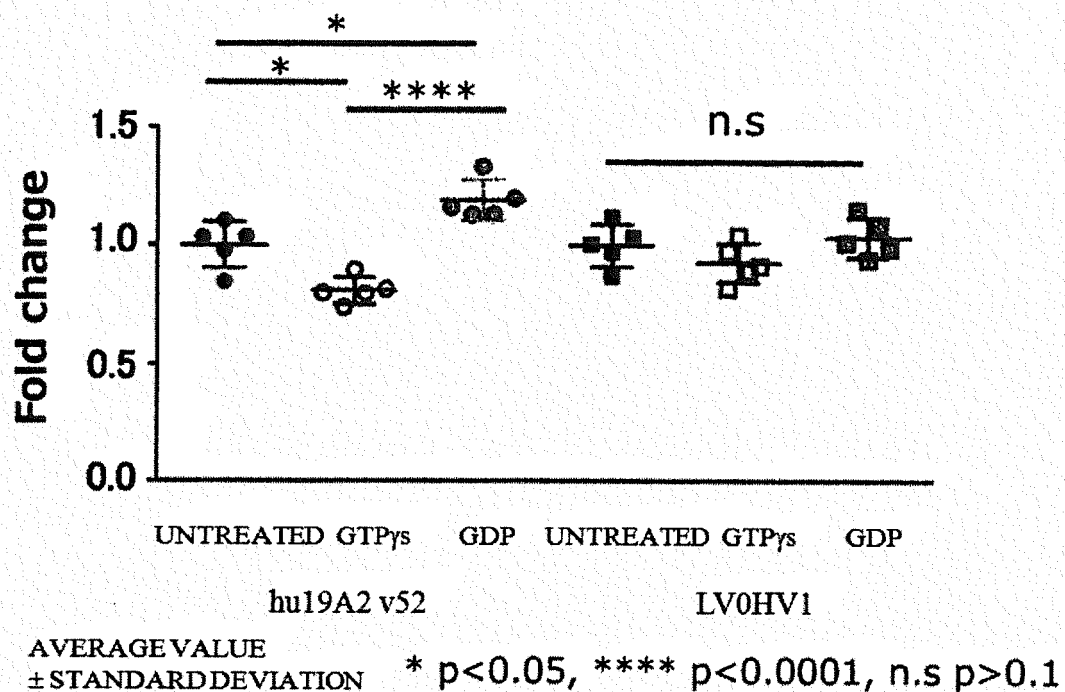
[FIG. 17]



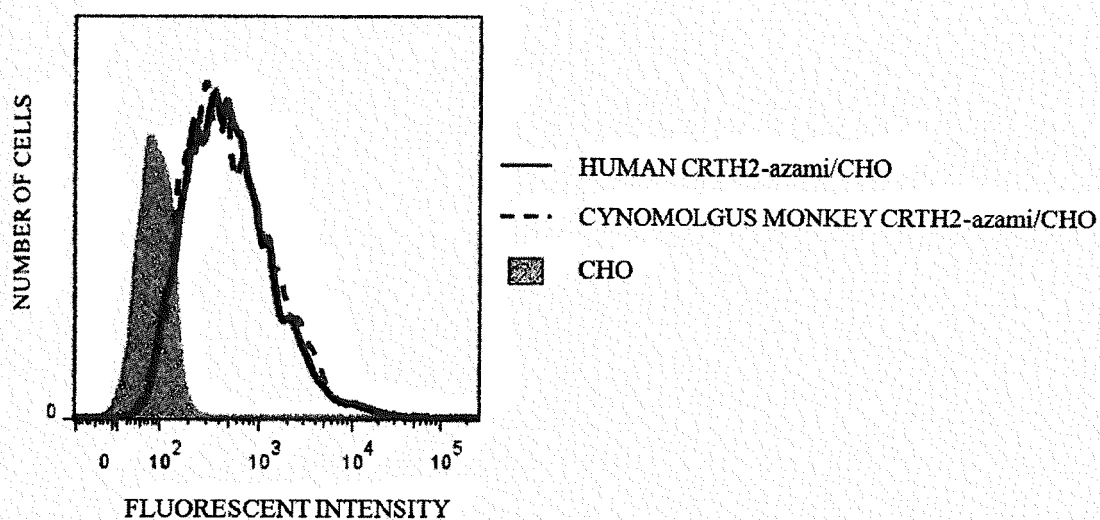
[FIG. 18]



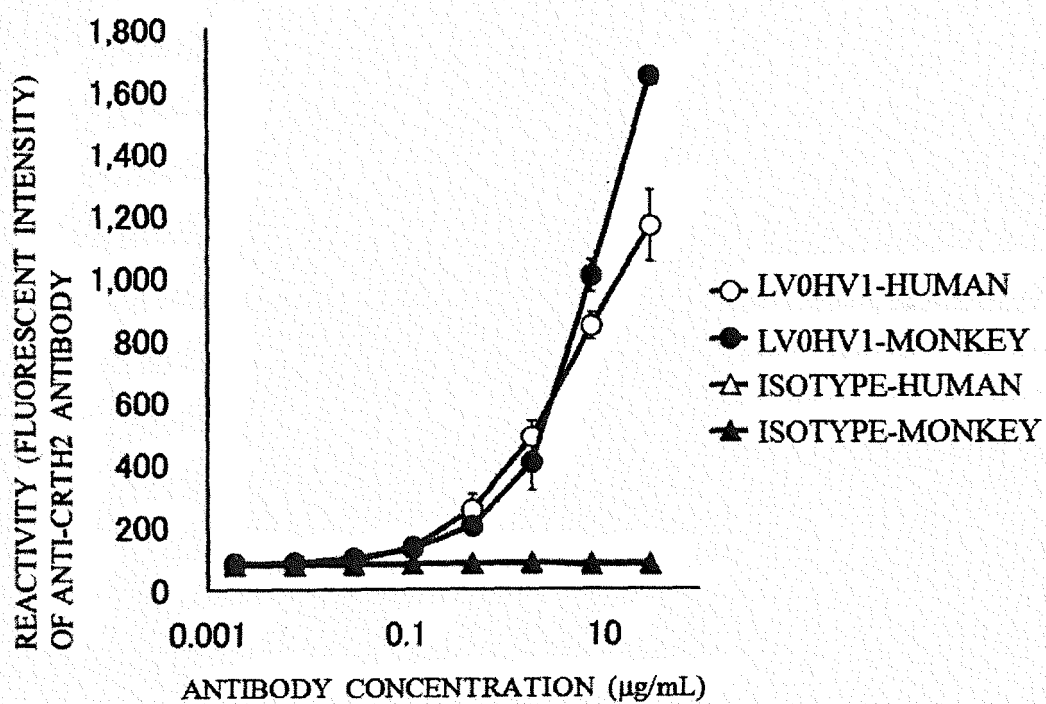
[FIG. 19]



[FIG. 20]



[FIG. 21]



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2016/071027

A. CLASSIFICATION OF SUBJECT MATTER
See extra sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K16/28, A61K39/395, A61P29/00, A61P37/02, A61P37/06, A61P37/08, C12N1/15, C12N1/19, C12N1/21, C12N5/10, C12N15/02, C12N15/09, C12P21/08, G01N33/53, G01N33/531

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2016
Kokai Jitsuyo Shinan Koho 1971-2016 Toroku Jitsuyo Shinan Koho 1994-2016

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

JSTPlus/JMEDPlus/JST7580(JDreamIII), CAlus/MEDLINE/EMBASE/BIOSIS(STN), WPIDS/WPIX(STN), GenBank/EMBL/DDBJ/GeneSeq, UniProt/GeneSeq

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	WO 2014/144865 A2 (GENENTECH, INC.), 18 September 2014 (18.09.2014), claims; examples; paragraphs [0004], [0026] to [0028], [0199] to [0202] & JP 2016-517441 A & US 2014/0328849 A1 & EP 2970471 A2	1-3, 5-26 4
Y A	NAGATA, K., et al., Selective expression of a novel surface molecule by human Th2 cells in vivo, J. Immunol., 1999, vol.162, no.3, p.1278- 1286, ISSN 0022-1767, particularly, Materials and Methods, p.1280	1-3, 5-26 4

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
04 October 2016 (04.10.16)

Date of mailing of the international search report
11 October 2016 (11.10.16)

Name and mailing address of the ISA/
Japan Patent Office
3-4-3, Kasumigaseki, Chiyoda-ku,
Tokyo 100-8915, Japan

Authorized officer

Telephone No.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2016/071027

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y A	"CRTH-2/GPR44 Antibody FAB33381N", Novus Biologicals [online], 2015-06-26 Updated, [retrieved on 2016.10.04], Retrieved from the Internet: <URL: http://www.funakoshi.co.jp/ data/datasheet/NOV/FAB33381N.pdf >, entire text	1-3, 5-26 4
Y A	"Human CRTH-2 Antibody", R&D Systems, Inc. [online], 2015-03-13, [retrieved on 2016.10.04], Retrieved from the Internet: <URL: http://www. funakoshi.co.jp/data/datasheet/RSD/MAB3338.pdf >, entire text	1-3, 5-26 4
A	WO 2005/093092 A2 (BAYER HEALTHCARE AG), 06 October 2005 (06.10.2005), claims; examples (Family: none)	1-26

Form PCT/ISA/210 (continuation of second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2016/071027

Continuation of A. CLASSIFICATION OF SUBJECT MATTER

(International Patent Classification (IPC))

C07K16/28(2006.01)i, A61K39/395(2006.01)i, A61P29/00(2006.01)i,
A61P37/02(2006.01)i, A61P37/06(2006.01)i, A61P37/08(2006.01)i,
C12N1/15(2006.01)i, C12N1/19(2006.01)i, C12N1/21(2006.01)i,
C12N5/10(2006.01)i, C12N15/02(2006.01)i, C12N15/09(2006.01)i,
C12P21/08(2006.01)i, G01N33/53(2006.01)i, G01N33/531(2006.01)i

(According to International Patent Classification (IPC) or to both national
classification and IPC)

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摘要

本发明涉及：特异性识别并结合人 CRTH2 的抗人 CRTH2 抗体；该抗体片段；编码该抗体的氨基酸序列的 DNA；含有该 DNA 的载体；生产该抗体的细胞和生产该抗体的杂交瘤；该抗体的制造方法；包含该抗体或抗体片段的组合物；使用该抗体或抗体片段用于如下疾病的治疗方法和诊断方法，例如过敏性疾病、自身免疫疾病、伴有嗜酸性粒细胞增多和功能亢进有关的疾病，以及与 Th2 细胞的增多和/或功能亢进有关的疾病；和包含该抗体或抗体片段的药物和诊断剂。