(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 July 2016 (28.07.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/118832 A1

(51) International Patent Classification:
C12N 5/0783 (2010.01) *C12N 5/0789* (2010.01)

(21) International Application Number:
PCT/US2016/014477

(22) International Filing Date:
22 January 2016 (22.01.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/106,507 22 January 2015 (22.01.2015) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) Title: CANCER IMMUNOTHERAPY

(57) Abstract: Methods of providing populations of NKT and/or γδ T cells, and their use, e.g., in therapies such as cancer immunotherapy.

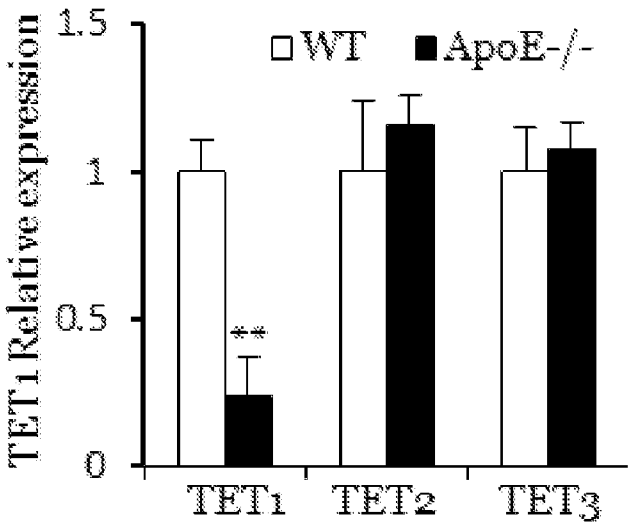


FIG. 1A

WO 2016/118832 A1

CANCER IMMUNOTHERAPY

CLAIM OF PRIORITY

This application claims the benefit of U.S. Patent Application Serial No. 62/106,507, filed on January 22, 2015. The entire contents of the foregoing are hereby incorporated by reference.

TECHNICAL FIELD

The invention relates, at least in part, to methods of providing populations of NKT and/or γδ T cells for use in tumor immunotherapy.

BACKGROUND

Cancer immunotherapies trigger the body's own immune system to find and destroy neoplastic cells. Natural killer T cells (NKT) and γδ T cells have been identified as critical components in cancer immunosurveillance. The initial success of preclinical trials in the last decades has evoked NKT or γδ T cells based immunotherapeutic approaches for the treatment of cancer. However, a significant proportion of patients are not eligible for NKT or γδ T cells based therapies because they don't have either a sufficient number of NKT or γδ T cells and/or lack sufficient cells with normal function. Although stem cell research has found that embryonic stem cells and induced pluripotent stem cells differentiate into NKT and γδ T cells, serving as a potential resource for clinical therapy, their differentiation efficiency is extremely low.

SUMMARY

The present invention is based, at least in part, on the discovery that Tet1 is essential to the differentiation of HSCs toward NKT and/or γδ T cells as well as their function, and that overexpressing Tet1 in hematopoietic stem cells (HSCs) increased their differentiation towards natural killer T cells (NKT) and gamma delta T cells (γδ T cells), e.g., by 10-20 times, in both in vivo and in vitro conditions. In addition, when Tet1 was overexpressed in HSCs they generated not only increased numbers of NKT and γδ T cells, but the cells that were generated were functionally superior in

their capacity to kill tumor cells, as injection of WT HSCs that overexpress Tet1 eliminated all of the carcinoma stages of neoplasia.

Thus, in a first aspect the present invention provides methods for preparing a population of Natural Killer T cells (NKT) and/or γδ T cells. The methods include obtaining a first population comprising hematopoietic stem cells (HSC); engineering the HSC to express (i.e., overexpress) Ten eleven translocation (Tet)1; maintaining the Tet1-overexpressing HSC in culture under conditions and for a time sufficient for at least some of the HSC to differentiate into NKT and/or γδ T cells; and optionally purifying the NKT and/or γδ T cells, thereby providing a population of NKT and/or γδ T cells.

Also provided herein is a population of NKT and/or γδ T cells prepared by a method described herein.

In another aspect, the invention provides populations of HSC engineered to overexpress Tet1, e.g., to express exogenous Tet1 or to overexpress endogenous Tet1 to produce levels of Tet1 above those found in normal, non-engineered cells; in some embodiments, the HSCs comprise a Tet1 gene operably linked to a regulatory region other than the endogenous Tet1 regulatory region.

In another aspect, the invention provides methods for treating a subject who has cancer. The methods include administering to the subject a population of NKT and/or γδ T cells described herein, or a population of HSC described herein.

In a further aspect, the invention provides methods for treating a subject who has cancer. The methods include obtaining a first population comprising hematopoietic stem cells (HSC); engineering the HSC to express Ten eleven translocation (Tet)1; and administering the Tet1-overexpressing HSC to the subject, thereby treating the subject.

In yet another aspect, the invention includes methods for treating a subject who has cancer. The methods include obtaining a first population comprising hematopoietic stem cells (HSC); engineering the HSC to overexpress Ten eleven translocation (Tet)1; maintaining the Tet1-expressing HSC in culture under conditions and for a time sufficient for at least some of the HSC to differentiate into NKT and/or γδ T cells; optionally purifying the NKT and/or γδ T cells, and administering the differentiated or purified population of NKT and/or γδ T cells to the subject, thereby treating the subject.

In some embodiments, the first population of HSC is obtained from the subject who has cancer. In some embodiments, the subject has colon cancer, ovarian cancer, prostate cancer, lymphoid malignancies, myeloma, renal cell carcinoma, breast cancer, or malignant glioma, or any cancer sensitive to immunosurveillance.

In an additional aspect, the invention provides methods for increasing levels of NKT and/or γδ T cells in a subject. The methods include obtaining a first population comprising hematopoietic stem cells (HSCs); engineering the HSCs to overexpress Ten eleven translocation (Tet)1; and administering the Tet1-expressing HSC to the subject, thereby increasing levels of NKT and/or γδ T cells in the subject.

In another aspect, the invention provides methods for increasing levels of NKT and/or γδ T cells in a subject. The methods include obtaining a first population comprising hematopoietic stem cells (HSCs); engineering the HSCs to overexpress Ten eleven translocation (Tet)1; maintaining the Tet1-expressing HSC in culture under conditions and for a time sufficient for at least some of the HSC to differentiate into NKT and/or γδ T cells; optionally purifying the NKT and/or γδ T cells, and administering the population of NKT and/or γδ T cells to the subject, thereby increasing levels of NKT and/or γδ T cells in the subject.

In some embodiments, the first population of HSCs is obtained from the subject.

In some embodiments of the methods described herein, the subject has a tumor.

In some embodiments of the methods described herein, the subject has carcinoma, sarcoma, myeloma, leukemia, or lymphoma. In some embodiments of the methods described herein, the subject has colon cancer, ovarian cancer, prostate cancer, lymphoid malignancies, myeloma, renal cell carcinoma, breast cancer, or malignant glioma.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their

entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

BRIEF DESCRIPTION OF DRAWINGS

Figures 1a-j. Hypercholesterolemia induced oxidant stress downregulates the expression of TET1 in HSCs that impairs their differentiation towards NKT and γδT cells. a, The expression of TET1, TET2 and TET3 in HSCs from WT and $ApoE^{-/-}$ mice. n=6, **, $p<0.01$, *vs.* WT. b, Oxidant stress dependent downregulation of TET1 expression in HSCs from $ApoE^{-/-}$ mice. n=6, *<0.05; **, $p<0.01$, *vs.* $ApoE^{-/-}$. c, The deletion of TET1 in HSCs. n=6, *, $p<0.05$; **, $p<0.01$, *vs.* WT; #, $p<0.05$, *vs.* $ApoE^{-/-}$. d, The differentiation of HSCs towards NKT *in vitro*. n=6, *, $p<0.05$; **, $p<0.01$, *vs.* WT; #, $p<0.05$, *vs.* $ApoE^{-/-}$. e. The differentiation of HSCs towards γδT cells *in vitro*. n=6, *, $p<0.05$; **, $p<0.01$, *vs.* WT; #, $p<0.05$, *vs.* $ApoE^{-/-}$. f, The overexpression of TET1 in HSCs. n=6, *, $p<0.05$; **, $p<0.01$, *vs.* WT; #, $p<0.05$; ##, $p<0.01$, *vs.* $ApoE^{-/-}$. g, The differentiation of HSCs towards NKT *in vitro*. n=6, *, $p<0.05$; **, $p<0.01$, *vs.* WT; #, $p<0.05$, *vs.* $ApoE^{-/-}$. h. The differentiation of HSCs towards γδT cells *in vitro*. n=6, *, $p<0.05$; **, $p<0.01$, *vs.* WT; #, $p<0.05$, *vs.* $ApoE^{-/-}$. i, The differentiation of HSCs towards NKT cells in vivo. n=6, *, $p<0.05$; **, $p<0.01$, *vs.* WT; #, $p<0.05$, *vs.* $ApoE^{-/-}$. j. The differentiation of HSCs towards γδT cells in vivo. n=6, *, $p<0.05$; **, $p<0.01$, *vs.* WT; #, $p<0.05$, *vs.* $ApoE^{-/-}$.

Figures 2a-e. The overexpression of TET1 alters the frequency of immature populations and specific subsets of NKT and γδT cells *in vitro*. a, HAS expression in NKT derived from *in vitro* co-culture. n=6, *, $p<0.05$, *vs.* WT+mock; #, $p<0.05$, *vs.* $ApoE^{-/-}$+mock. b, HAS expression in γδT cells derived from *in vitro* co-culture. n=6, *, $p<0.05$, *vs.* WT+mock; #, $p<0.05$, *vs.* $ApoE^{-/-}$+mock. c,d,e. V1 (c), V2 (d) and V6 (e) subsets in γδT cells derived from *in vitro* co-culture of TET1 overexpressing HSCs. n=6, *, $p<0.05$, *vs.* WT+mock; #, $p<0.05$, *vs.* $ApoE^{-/-}$+mock.

Figures 3a-g. The overexpression of TET1 alters the frequency of immature population and specific subsets of NKT and γδT cells in vivo. a, HAS expression in NKT derived from *in vitro* co-culture. n=6, *, $p<0.05$, *vs.* WT+mock; #, $p<0.05$, *vs.* $ApoE^{-/-}$+mock. b, HAS expression in γδT cells derived from *in vitro* co-culture. n=6, *, $p<0.05$, *vs.* WT+mock; #, $p<0.05$, *vs.* $ApoE^{-/-}$+mock. c,d,e. V1 (c), V2 (d) and V6

(e) subsets in γδT cells derived from *in vitro* culture of TET1 overexpressing HSCs. n=6, *, p<0.05, *vs.*. WT+mock; #, p<0.05, *vs.* ApoE$^{-/-}$+mock. f. CCR6^{+} population in γδT cells derived from recipient mice; g, IL-17^{+} cells in γδT cells derived from recipient mice. n=8, *, p<0.05, *vs.*. WT+mock; #, p<0.05, *vs.* ApoE$^{-/-}$+mock.

Figure 4. The overexpression of TET1 alters the differentiation of CD4^{+} and CD8^{+} populations in *in vitro* co-culture of HSCs. n=8, *, p<0.05, *vs.* WT+mock; #, p<0.05, *vs.* ApoE$^{-/-}$+mock.

Figures 5a-f. Reconstitution of lethally irradiated WT mice with ApoE$^{-/-}$ HSCs that overexpresses TET1 restores immunosurveillance against colorectal neoplasia. a, The frequency of NKT cells in thymus and blood of the recipients after transplantation with WT HSCs, ApoE$^{-/-}$ HSCs, TET1-overexpressing WT HSCs+WT HSCs, or TET1-overexpressing ApoE$^{-/-}$ HSCs+ApoE$^{-/-}$ HSCs. n=8, *, p<0.05, *vs.* WT-WT; #, p<0.05, *vs.*. ApoE$^{-/-}$-WT. b. The frequency of γδ T cells in thymus and blood of the recipients. n=8, *, p<0.05, *vs.* WT-WT; #, p<0.05, *vs.* ApoE$^{-/-}$-WT. c, The frequency of NKT cells in colon of the recipients. n=8, **, p<0.01, *vs.* WT-WT; #, p<0.05, *vs.*. ApoE$^{-/-}$-WT. d, The frequency of γδ T cells in colon of the recipients. n=8, **, p<0.01, *vs.* WT-WT; #, p<0.05, *vs.*. ApoE$^{-/-}$-WT. e, Average tumor numbers per mouse in the recipients. n=12, *, p<0.05, *vs.* WT-WT; #, p<0.05, *vs.* ApoE$^{-/-}$-WT. f, Histopathologic stages of tumors. n=12, *, p<0.05, **, p<0.01 *vs.* WT-WT; #, p<0.05, ##, p<0.01, *vs.* ApoE$^{-/-}$-WT.

DETAILED DESCRIPTION

Natural killer T (NKT) cells, defined by the expression of both αβ T-cell receptors (TCR) and lineage markers of natural killer (NK) cells, are a small population of lymphocytes that possess characteristics of both innate and adaptive immune cells (1,2). Upon activation, NKT and γδT cells rapidly secrete a variety of cytokines, including interferon γ (IFNγ), interleukins (IL)- 4, IL-13, IL-17, tumor necrosis factor α (TNFα), and granulocyte macrophage colony-stimulating factor (GM-CSF) (Hayday, Annu Rev Immunol. 18, 975-1026 (2000); Brennan et al., Nat Rev Immunol. 13, 101-17 (2013)). Along with the mediators produced by antigen-presenting cells with which NKT and γδT cells interact, these cytokines recruit and stimulate the anti-tumor functions of cytotoxic lymphocytes, boosting innate as well as adaptive antitumor responses. Activated NKT and γδT cells both have strong cytotoxic effector activity (Chien et al., Annu Rev Immunol. 32, 121-55 (2014);

Taniguchi et al., Nat Immunol. 4, 1165-1165 (2003); Todaro et al., J Immunol. 182, 7287-7296 (2009)). In this context, NKT and γδT cells function as major participants in tumor immunosurveillance. Recent studies showed that iNKT-deficient mice exhibited significantly increased susceptibility to methylcholanthrene-(MCA) induced sarcomas and B16F10 melanoma tumors (3), an effect reversed by the administration of liver-derived iNKT cells during the early stages of tumor growth (4). Interferon (IFN)-γ production by NKT cells has also been shown to be critical in tumor rejection. The primary contribution of NKT cells to tumor immunosurveillance occurs indirectly via the activation of NKT cells by dendritic cells (DC) presenting alpha-galactosylceramide (α-GalCer). Activated NKT cells then initiate a series of cytokine cascades that help boost the priming phase of the antitumor immune response. These studies indicate that NKT cells are an essential component in the immunosurveillance against cancers.

T lymphocytes bearing γ- and δ-chain T-cell receptor heterodimers are named γδ T cells and have been identified as another important cellular component in the immunosurveillance against cancer. Antigen recognition of γδ T-cell receptors is very unique, and the responses frequently exhibit innate characteristics. Furthermore, peripheral γδ T cells exert a number of effector and regulatory functions (5). γδ T cells rapidly produce cytokines like IFN-γ and IL-17 and promote inflammation, partly due to their inherent epigenetic and transcriptional programs, which facilitates a rapid and comprehensive killing response to neoplastic cells. Moreover, γδ T cells lyse target cells directly, which is necessary for pathogen or tumor clearance (6).

Recent studies have shown that NKT and γδ T cells could be steadily expanded *in vitro* and employed in cancer immunotherapy. Clinical trials have been completed in a cohort of 17 patients with advanced non-small cell lung cancers and 10 cases of head and neck tumors. Sixty percent of advanced lung cancer patients with high IFN-γ production had significantly prolonged median survival times of 29.3 months with only the primary treatment. In the case of head and neck tumors, 10 patients who completed the trial all had stable disease or partial responses five weeks after the combination therapy of α-GalCer-DCs and activated NKT cells. Cancer immunotherapy trials with autologous γδ T cells have been investigated in parallel by Japanese, Australian and French groups. Their results suggested that γδ T cells based

therapy is well tolerated and therapeutically effective, as many patients showed stabilized diseases following this treatment (7, 8, 9, 10).

Based on the initial success in preclinical trials, intense efforts have been made in the last decades to launch NKT or γδ T cells based immunotherapeutic approaches for the treatment of cancer. However, a significant proportion of patients are not eligible for NKT or γδ T cells based therapies because they don't have sufficient NKT and/or γδ T cells (11, 12). Although stem cell research has provided evidence that embryonic stem cells and induced pluripotent stem cells differentiated into NKT and γδ T cells in vitro, serving as a potential resource for clinical therapy, the differentiation efficiency is questionable (12,13). Therefore, it is a priority goal in NKT or γδ T cell based cancer immunotherapy to establish an adequate and reliable resource of these cells.

Enhancing Hematopoietic Stem Cell Differentiation toward NKT and γδ T cells

Described herein are methods for creating populations of NKT and γδ T cells by overexpressing Ten eleven translocation (Tet)1 in hematopoietic stem cells. Members of the Tet protein family, including Tet1, Tet2 and Tet3, are ketoglutarate and Fe2+ dependent enzymes that can specifically modify DNA by demethylation (14,15,16). Within the Tet family, Tet2 has been shown to have a critical role in regulating the self-renewal, proliferation and differentiation of HSCs (14, 17), whereas the role of Tet1 in hematopoiesis was previously unknown. The present inventors found that Tet1-dependent epigenetic regulation is a novel determinant in the differentiation of hematopoietic stem cells (HSCs) towards NKT and γδT cells. Tet1 overexpression in HSCs dramatically increases the differentiation of HSCs towards NKT and γδT cells and restores the impaired immunosurveillance against colorectal cancer in hypercholesterolemic mice. Based on these findings, the present methods can be used to provide human NKT and γδ T cells for cancer immunotherapy by manipulating Tet1 dependent epigenetic regulation in HSCs.

Thus, the present methods include obtaining a first population of hematopoietic stem cells (HSC), preferably from an affected person. Preferably, the HSCs are obtained from a human subject who is going to receive the immunotherapy treatment with NKT and γδ T cells, i.e., the cells are autologous; alternatively, they can be allogeneic. Methods for obtaining enriched populations of HSC are known in the art and include cell sorting based on expression of one or more cell surface

markers; in some embodiments, the HSC used in the present methods are CD34+; in some embodiments, the cells are CD34+, Thy-1+; in some embodiments, the cells are CD34+, CD59+, Thy1/CD90+, CD38lo/-, C-kit/CD117+, and/or lin-. For example, primary human CD34+-enriched cells can be obtained from peripheral blood, e.g., after treatment of the donor with a mobilizing cytokine such as granulocyte-colony stimulating factor (GCSF). Other sources of HSC include bone marrow and umbilical cord blood. A number of methods are known in the art for preparing enriched populations of HSC, e.g., as described in Rector et al., Methods Mol Biol. 2013;976:1-15. For example, the cells can be sorted, e.g., using columns (e.g., the MiniMACS LS+ separation columns (Miltenyi Biotec, Auburn, CA)), e.g., using commercially available kits, e.g., the CD34 –progenitor cell isolation kit (StemCell Technologies, Vancouver, BC, Canada), according to the manufacturer's protocol. A population of cells that is enriched for HSCs is at least 20% HSC, e.g., is at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% HSCs. In some embodiments, the HSCs used in the present methods are obtained by enriching for cells that are CD34+; in some embodiments, the cells are obtained by enriching for cells that are CD34+, Thy-1+; in some embodiments, the cells are obtained by enriching for cells that are CD34+, CD59+, Thy1/CD90+, CD38lo/-, C-kit/CD117+, lin-.

Tet1

The enriched populations of HSCs used in the present methods and compositions are engineered to express the Tet1 protein. The sequence of human Tet1 is as follows:

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   1 msrsrharps rlvrkedvnk kkknsqlrkt tkganknvas vktlspgklk qliqerdvkk
  61 ktepkppvpv rsllltragaa rmnldrtevl fqnpesltcn gftmalrsts lsrrlsqppl
 121 vvakskkvpl skglekqhdc dykilpalgv khsendsvpm qdtqvlpdie tligvqnpsl
 181 lkgksqettq fwsqrvedsk inipthsgpa aeilpgpleg trcgeglfse etlndtsgsp
 241 kmfaqdtvca pfpqratpkv tsqgnpsiql eelgsrvesl klsdsyldpi ksehdcypts
 301 slnkvipdln lrnclalggs tsptsvikfl lagskqatlg akpdhqeafe atanqqevsd
 361 ttsflgqafg aiphqwelpg adpvhgealg etpdlpeipg aipvqqgevfg tildqqetlg
 421 msgsvvpdlp vflpvppnpi atfnapskwp epqstvsygl avqqgaiqilp lgsghtpqss
 481 snseknslpp vmaisnvene kqvhisflpa ntqgfplape rglfhaslgi aqlsqagpsk
 541 sdrgssqvsv tstvhvvntt vvtmpvpmvs tssssyttll ptlekkkrkr cgvcepcqqk
 601 tncgectyck nrknshqick krkceelkkk psvvvplevi kenkrpqrek kpkvlkadfd
 661 nkpvngpkse smdysrcghg eeqklelnph tvenvtkned smtgievekw tqnkksqltd
 721 hvkgdfsanv peaeksknse vdkkrtkspk lfvqtvrngi khvhclpaet nvsfkkfnie
 781 efgktlenns ykflkdtanh knamssvatd mscdhlkgrs nvlvfqqpgf ncssiphssh
 841 siinhhasih negdqpktpe nipskepkdg spvqpsllsl mkdrrltleq vvaiealtql
 901 seapsenssp sksekdeese qrtasllnsc kailytvrkd lqdpnlqgep pklnhcpsle
 961 kqsscntvvf ngqtttlsns hinsatnqas tksheyskvt nslslfipks nsskidtnks
1021 iaqgiitldn csndlhqlpp rnneveycnq lldsskklds ddlscqdath tqieedvatq
1081 ltqlasiiki nyikpedkkv estptslvtc nvqqkynqek gtiqqkppss vhnnhgsslt
1141 kqknptqkkt kstpsrdrrk kkptvvsyqe ndrqkwekls ymygticdiw iaskfqnfgq
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1201 fcphdfptvf gkissstkiw kplaqtrsim qpktvfpplt qiklqrypes aeekvkvepl
1261 dslslfhlkt esngkaftdk aynsqvqltv nanqkahplt qpssppnqca nvmagddqir
1321 fqqvvkeqlm hqrlptlpgi shetplpesa ltlrnvnvvc sggitvvstk seeevcsssf
1381 gtsefstvds aqknfndyam nfftnptknl vsitkdselp tcscldrviq kdkgpyythl
1441 gagpsvaavr eimenrygqk gnairieivv ytgkegkssh gcpiakwvlr rssdeekvlc
1501 lvrqrtghhc ptavmvvlim vwdgiplpma drlytelten lksynghptd rrctlnenrt
1561 ctcqgidpet cgasfsfgcs wsmyfngckf grspsprrfr idpssplhek nledmlqsla
1621 trlapiykqy apvayqnqve yenvarecrl gskegrpfsg vtacldfcah phrdihnmnn
1681 gstvvctltr ednrslgvip qdeqlhvlpl yklsdtdefg skegmeakik sgaievlapr
1741 rkkrtcftqp vprsgkkraa mmtevlahki ravekkpipr ikrknnsttt nnskpsslpt
1801 lgsntetvqp evksetephf ilkssdntkt yslmpsaphp vkeaspgfsw spktasatpa
1861 plkndatasc gfsersstph ctmpsgrlsg anaaaadgpg isqlgevapl ptlsapvmep
1921 linsepstgv tepltphqpn hqpsfltspq dlasspmeed eqhseadepp sdeplsddpl
1981 spaeeklphi deywsdsehi fldaniggva iapahgsvli ecarrelhat tpvehpnrnh
2041 ptrlslvfyq hknlnkpqhg felnkikfea keaknkkmka seqkdqaane gpeqssevne
2101 lnqipshkal tlthdnvvtv spyalthvag pynhwv                (SEQ ID NO:1)
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In some embodiments, the Tet1 proteins that are expressed in the enriched HSCs can be at least about 80%, 85%, 90%, 95%, 98% or more homologous to SEQ ID NO:1, and maintain the ability to promote HSC differentiation to NKT or γδ T cells. In some embodiments the Tet1 comprises the catalytic domain of Tet1, e.g., amino acids 1418–2136 of SEQ ID NO:1, or a sequence that is at least about 80%, 85%, 90%, 95%, 98% or more homologous to amino acids 1418–2136 of SEQ ID NO:1 and maintains the ability to promote HSC differentiation to NKT or γδ T cell. Another exemplary nucleic acid sequence encoding human Tet1 is in GenBank at Acc. No. NM_030625.2.

Recombinant Expression Vectors, Host Cells and Genetically Engineered Cells

Generally speaking, the HSC are engineered to express Tet1 by transduction with a nucleic acid, e.g., expression vectors, containing a nucleic acid encoding a Tet1 polypeptide described herein. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked and can include a plasmid, cosmid or viral vector. The vector can be capable of autonomous replication or it can integrate into a host DNA. Viral vectors include, e.g., replication defective retroviruses, recombinant retroviruses, adenovirus, adeno-associated virus, lentivirus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered naked or with the help of, for example, cationic liposomes (lipofectamine) or derivatized (e.g., antibody conjugated), polylysine conjugates, gramacidin S,

artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or CaPO4 precipitation carried out in vivo.

A preferred approach for in vivo introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, e.g., a cDNA. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells that have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors can be used as a recombinant gene delivery system for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors can provide effective delivery of genes into cells. Whereas the transgene within a retroviral vector is typically stably integrated into the chromosomal DNA of the host, the transgene of an AAV vector usually exists as extrachromosomal episomes within the cytoplasm of infected cells. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are characterized for use in gene transfer for gene therapy purposes (for a review see Miller, *Blood* 76:271 (1990)). A replication defective retrovirus can be packaged into virions, which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells in vitro or in vivo with such viruses can be found in Ausubel, et al., eds., *Current Protocols in Molecular Biology*, Greene Publishing Associates, (1989), Sections 9.10-9.14, and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ΨCrip, ΨCre, Ψ2 and ΨAm. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, in vitro and/or in vivo (see for example Eglitis, et al. (1985) Science 230:1395-1398; Danos and Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464; Wilson et al. (1988) Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano et al. (1990) Proc. Natl. Acad. Sci. USA 87:6141-6145; Huber et al. (1991) Proc. Natl. Acad. Sci. USA 88:8039-8043; Ferry et al. (1991) Proc. Natl. Acad. Sci. USA 88:8377-8381; Chowdhury et al. (1991) Science

254:1802-1805; van Beusechem et al. (1992) Proc. Natl. Acad. Sci. USA 89:7640-7644; Kay et al. (1992) Human Gene Therapy 3:641-647; Dai et al. (1992) Proc. Natl. Acad. Sci. USA 89:10892-10895; Hwu et al. (1993) J. Immunol. 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Another viral gene delivery system useful in the present methods utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated, such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See, for example, Berkner et al., BioTechniques 6:616 (1988); Rosenfeld et al., Science 252:431-434 (1991); and Rosenfeld et al., Cell 68:143-155 (1992). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, or Ad7 etc.) are known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances, in that they are not capable of infecting non-dividing cells and can be used to infect a wide variety of cell types, including epithelial cells (Rosenfeld et al., (1992) supra). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situ, where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al., supra; Haj-Ahmand and Graham, J. Virol. 57:267 (1986).

Yet another viral vector system useful for delivery of nucleic acids is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al., Curr. Topics in Micro. and Immunol.158:97-129 (1992). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al., Am. J. Respir.

Cell. Mol. Biol. 7:349-356 (1992); Samulski et al., J. Virol. 63:3822-3828 (1989); and McLaughlin et al., J. Virol. 62:1963-1973 (1989). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al., Mol. Cell. Biol. 5:3251-3260 (1985) can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al., Proc. Natl. Acad. Sci. USA 81:6466-6470 (1984); Tratschin et al., Mol. Cell. Biol. 4:2072-2081 (1985); Wondisford et al., Mol. Endocrinol. 2:32-39 (1988); Tratschin et al., J. Virol. 51:611-619 (1984); and Flotte et al., J. Biol. Chem. 268:3781-3790 (1993).

Typically, an expression vector includes the nucleic acid in a form suitable for expression of the human Tet1 in an HSC. Preferably the recombinant expression vector includes one or more regulatory sequences operatively linked to the nucleic acid sequence to be expressed. The term "regulatory sequence" includes promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence, as well as tissue-specific regulatory and/or inducible sequences. The design of the expression vector can depend on such factors as the level of expression of protein desired and whether regulated or inducible expression is desired. The expression vectors can be introduced into HSCs. The expression vector is preferably a vector suitable for expression in mammalian cells, and the expression vector's control functions can be provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. See, e.g., Wang et al., Exp Hematol. 2008 Jul;36(7):823-31.

In another aspect the invention provides HSC that include and optionally express a Tet1 nucleic acid molecule described herein, e.g., a Tet1 nucleic acid molecule within a recombinant expression vector or a Tet1 nucleic acid molecule containing sequences which allow it to homologously recombine into a specific site of the HSC's genome. The term HSC refers not only to the particular subject cell that is transduced but to the progeny or potential progeny of such a cell that contain the Tet1 nucleic acid. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be

identical to the parent cell, but are still included within the scope of the term as used herein.

Vector DNA can be introduced into host cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation.

In another aspect, the invention features an HSC cell or purified preparation of HSCs that include a Tet1 transgene, which over-express Tet1 or express Tet1 in response to a stimulus.

Also provided herein are human hematopoietic stem cells, in which an endogenous Tet1 is under the control of an exogenous regulatory sequence that does not normally control the expression of the endogenous Tet1 gene, and that express Tet1 under circumstances in which a cell that lacks the exogenous regulatory sequence do not express Tet1. The expression characteristics of an endogenous Tet1 gene within a cell can be modified by inserting a heterologous DNA regulatory element into the genome of the cell such that the inserted regulatory element is operably linked to the endogenous Tet1 gene. For example, an endogenous Tet1 gene that is "transcriptionally silent," e.g., not normally expressed, or expressed only at very low levels, may be activated by inserting a regulatory element capable of promoting the expression of a normally expressed gene product in that cell. Techniques such as targeted homologous recombination can be used to insert the heterologous DNA as described in, e.g., Chappel, US 5,272,071; WO 91/06667, published in May 16, 1991.

The methods can also include identifying, selecting, and/or purifying those cells that overexpress Tet1, or that express Tet1 over a desired level.

The Tet1-expressing cells can be used for administration to a subject, can be frozen or otherwise stored for later administration to a subject, or can be maintained under conditions such that the HSC differentiate into NKT and γδ T cells. These conditions can include those previously described. For example, c-kit+ Sca-1+ Lin- (KSL) hematopoietic stem cells can be seeded, e.g., at 4×10^3 cells/well into 12-well tissue culture plates, containing a confluent monolayer of OP9-DL1 cells; see, e.g.,

Holmes and Zuniga-Pflucker, Cold Spring Harb Protoc 2009: oi:10.1101/pdb.prot5156 (2009)). In some embodiments, the cultures are performed in the presence of one or more cytokines or growth factors, e.g., 5 ng/mL IL-2, 10 ng/mL GM-CSF (Stem cell Technology), 5 ng/mL, IL-7, and 5 ng/mL mFLT3 (Peprotech).

NKT cells can be identified by methods known in the art, e.g., by the presence of TCRαβ and NK1.1 or CD1d-tet (see, e.g. Godfrey et al,. Nature Reviews Immunology 4, 231-237 (2004)); γδ T cells can be identified by methods known in the art, e.g., by the presence of γδ TCR (see, e.g., Holtmeier and Kabelitz, Chemical Immunology and Allergy 86: 151–83 (2005)). The cells can be maintained in culture until a desired number of cells, e.g., of HSC or NKT and γδ T cells, is obtained, and then harvested for use or freezing. The methods can also include purifying the NKT and/or γδ T cells away from the Tet1-expressing HSC, to provide purified populations of NKT and/or γδ T cells.

Methods of Targeting Neoplasias

The present methods include the use of enriched populations of Tet1-epxressing HSC, or NKT and γδ T cells obtained from Tet1-expressing HSC, for treating a neoplasia, e.g., a tumor, in a subject. As noted in Bennouna et al., Cancer Immunol Immunother (2010) 59:1521–1530, "An expansive body of literature in the field has documented that cd T cells, which represent 1–10% of human peripheral T cells, kill solid and hematologic tumors originating from virtually any organ type." NKT and γδ T cells have been shown to be effective in treating a wide range of lymphoid malignancies as well as solid tumor-associated cancers, including colon cancer, colorectal cancer; gastrointestinal carcinoma, hepatocarcinoma, esophageal cancer, ovarian cancer, prostate cancer, myeloma, renal cell carcinoma, breast cancer, non-small cell lung cancer, and malignant glioma, among others, see, e.g., Fisher et al., Oncoimmunology. 2014; 3: e27572; Kobayashi et al., Anticancer Research 31: 1027-1032 (2011); Motohashi et al., Clin Cancer Res 2006;12:6079-6086; Bennouna et al., Cancer Immunol Immunother (2010) 59:1521–1530; and Kobayashi et al., Cancer Immunol Immunother (2007) 56:469–476.

Thus the present methods can include identifying a subject who has a neoplasm, e.g., a tumor, and administering to the subject a therapeutically effective amount of a population of Tet1-expressing HSC, or NKT and/or γδ T cells obtained

from Tet1-expressing HSC. In some embodiments, the Tet1-expressiong HSC, NKT and/or γδ T cells are prepared by a method described herein from a population of the subject's own (autologous) HSC; in some embodiments, the HSC are obtained from a related or unrelated type-matched donor. In some embodiments, the neoplasm is a tumor, e.g., a tumor that is sensitive to innate immunity against cancer or immunosurveillance, e.g., carcinoma, sarcoma, myeloma, leukemia, or lymphoma. In some embodiments, the methods include determining a level of native NKT and/or γδ T cells in the subject, comparing the level of NKT and/or γδ T cells to a reference level (e.g., a level of NKT and/or γδ T cells determined, based on analysis of a cohort of subjects, to correlate to a level of NKT and/or γδ T cells in subjects who would benefit from the administration of additional NKT and/or γδ T cells, e.g., subjects who are deficient in native NKT and/or γδ T cells). The levels of NKT and/or γδ T cells can be measured, e.g., in the circulating blood, in the thymus, and/or in a tumor in the subject.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Materials and Methods

The following materials and methods were used in the examples set forth herein.

Mice

All mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were maintained in the mouse barrier facility. Care of mice was in accordance with NIH guidelines and the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School approved all protocols. Mice were kept on a 12 hr day/night schedule and were allowed free access to chow and water. $ApoE^{-/-}$ and WT mice were fed standard mouse chow (5.4 g fat/100 g diet, 0% cholesterol). HCD mice were fed a diet with 10 g fat/100 g diet, 11.25 g cholesterol/100 g diet (Research Diets, New Brunswick, NJ). NAC was given for 8 weeks (150mg/kg/day via drinking water).

Tumor induction and analysis

The colorectal neoplasia were performed as described in previous publications (Greten et al., Cell. 118(3), 285-96 (2004)). Three month old mice were subcutaneously injected with a solution of Azoxymethane (AOM) at a dose rate of 15 mg/kg body weight, once weekly for 3 successive weeks. 2% DSS was given in the drinking water over five days in the last week. Mice were sacrificed ten weeks after the last injection of AOM. Colons were removed and flushed with PBS. Sections (5 μm) were cut stepwise (200 μm) through the complete block and stained with H&E. Tumors counts were performed in a blinded fashion. To determine the histopathologic stages of tumors, the sections of tumors were read by cancer pathologists in a blind fashion.

Flow cytometry

Cells were stained with monoclonal antibodies conjugated to various fluoroprobes. These antibodies included: cKit (2B8), Sca-1 (E13-161.7), CD4 (L3T4), CD8 (53-6.72), CD90.1, CD25, CD44, TCRβ, NK1.1, γδTCR, CD45.1, CD45.2. The lineage cocktail consisted of CD4, CD8, B220 (RA3-6B2), TER-119, Mac-1 (MI/70), and Gr-1 (RB6-8C5). All antibodies were purchased from BD Bioscience (San Diego, CA). CD1d-aGalCer tetramer was obtained from the NIH Tetramer facility. FACS analysis was carried out on a FACS Diva or MoFlow.

Lentiviral particle preparation and transduction

The Tet1 specific and control shRNA plasmids were both purchased from Santa Cruz (CA, USA). The plasmid with TET1 catalytic domain (pTYF-U6-shCONT-EF1-Puro-2A-CD1) was a gift from Dr Yi Zhang (Massachusetts General Hospital, Boston, MA). The envelope and helper plasmids were purchased from ABM (Toronto, Canada). The lentiviral particles were prepared according to the kit instruction. The lentivirus-containing supernatant was harvested 2 days post-transfection. Fresh isolated KSL cells were transduced with lentivirus for 24 hours and then selected with puromycin (2μg/ml) (Santa Cruz Biotechnology, CA, USA) for 72 hours.

HSCs and OP9 Cell Co-culture

The co-culture was performed as described (e.g., Holmes and Zuniga-Pflucker, Cold Spring Harb Protoc 2009: oi:10.1101/pdb.prot5156 (2009)). KSL cells were seeded at 4×10^3 cells/well into 12-well tissue culture plates containing a

confluent monolayer of OP9-DL1 cells. OP9-DL1 cells were a kind gift from Dr. Juan Carlos Zuniga-Pflucker (University of Toronto). All cultures were performed in the presence of 5ng/mL IL-2, 10ng/mL GM-CSF (Stem cell Technology), 5ng/mL, IL-7, 5ng/mL mFLT3 (Peprotech). Co-cultures were harvested by forceful pipetting at the indicated time points.

Immunohistochemistry

We used a standard protocol to detect NKT and γδ T cells in colon and tumor tissues. The antibodies were purchased from BD Biosciences (MA, USA). For indirect immunohistochemistry, we used rabbit-specific IgG conjugated with FITC or PE (Chemicon) as a secondary antibody. For nuclear staining, we treated specimens with DAPI (Molecular Probes). Fluorescent images were obtained using a confocal laser scanning microscope (Carl Zeiss LSM 510 system; Carl Zeiss).

Analysis of intracellular ROS

We loaded samples of cultures with DCF-DA (Sigma) and incubated them on a shaker at 37 °C for 30 min. The peak excitation wavelength for oxidized DCF was 488 nm, and emission was 525 nm. The concentration of H2O2 was measured by Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes).

Chromatin Immunoprecipitation (ChIP)

ChIP was performed with minor modifications of the procedure described by Yildirim et al. (Nature Structural & Molecular Biology 19:56–61 (2012)). Approximately 6×10^6 Hepa-1 cells were incubated for 10 min at room temperature with 1% formaldehyde. After cross-linking, the reaction was quenched with 0.25 M glycine for 10 min at room temperature. Proteins are initially cross-linked to DNA and nuclei are pelleted and sonicated to 200-500 bp fragments (Bioruptor, Diagenode). The cross-linked DNA was immunoprecipitated with H3K4me3 or H3K27me3 antibodies (Millipore, USA) overnight at 4 °C with rotation, DNA-Antibody complexes were bound to ChIP beads, pulled down, washed and then eluted from beads. Following reversal of cross-linkage purified DNA was used for Quantitative PCR using ChIP PCR primers which were purchased from IDT (MA, USA). Immunoprecipitation efficiency was calculated by normalizing sample C_T values against control IgG values and calculating ratios of sample C_T values relative to input values.

RT–PCR and qRT-PCR array

We reverse transcribed cDNAs from total RNA isolated from each cell fraction using Trizol LS (Invitrogen). Transcription to cDNA was performed using SuperScript III (Invitrogen). All PCRs were carried out in triplicate using an Eppendorf Mastercycler (Eppendorf).

DNA extraction, bisulfite conversion and pyrosequencing

Genomic DNA was extracted from freshly isolated cells using QIAamp DNA MiniKit (Qiagen Inc., Hilden, Germany) and quantified by UV absorption. 200–300 ng of DNA was used in the bisulfite conversion reactions where unmethylated cytosines were converted to uracil with the Epi-Tect Bisulfite kit (Qiagen) according to manufacturer's instructions. Briefly, DNA was mixed with water, DNA protect buffer and bisulfite mix and the conversion was run in a thermocycler (Biometra, Goettingen, Germany) at the recommended cycle conditions. Converted DNA was purified on a spin column and eluted twice into a total of 40 μl Buffer EB.

PCR and pyrosequencing

Primer sets with one biotin-labelled primer were used to amplify the bisulfite converted DNA. New primers for each gene were designed using PyroMark Assay Design software version 2.0.1.15 (Qiagen). The size of the amplicons was restricted to a maximumof 210 bp. Due care was taken to avoid any primer overlapping CG dyads to prevent amplification biases.

We examined at least two different sites within the CpG islands separated by several hundred base pairs. To provide the internal control for total bisulfite conversion, a non-CG cytosine in the region for pyrosequencing was included where possible. PCRs were performed using a converted DNA equivalent of 200 cells employing the PyroMark PCR kit (Qiagen). The cell genome-equivalents of DNA calculations assumed 6 pg DNA per diploid cell. Briefly, 12.5 μl master mix, 2.5 μl Coral red, 5pmol of each primer, 7 μl of water and 2 μl sample were mixed for each reaction and run at thermal cycling conditions: 95◦C for 15 min and then 45 cycles: 30 sec at 94◦C; 30 sec at the optimized primer-specific annealing temperature; 30 sec at 72◦C and a final extension for 10 min at 72◦C. The amplified DNA was confirmed by electrophoresis in a 2% low melting point agarose gel (Sigma-Aldrich, Steinheim, Germany). 3 μl streptavidin beads (GE Healthcare, Buckinghamshire, UK), 37 μl PyroMark binding buffer (Qiagen), 20 μl PCR product and 20 μl water were mixed

and incubated for 10 min on a shaking table at 1300 rpm. Using the Biotage Q96 Vaccum Workstation, amplicons were separated, denatured, washed and added to 45 μl annealing buffer containing 0.33 μM of pyrosequencing primer. Primer annealing was performed by incubating the samples at 80◦C for 2 min and allowed to cool to room temperature prior to pyrosequencing. PyroGold reagents were used for the pyrosequencing reaction and the signal was analyzed using the PSQ 96MA system (Biotage, Uppsala, Sweden). Target CGs were evaluated by instrument software (PSQ96MA 2.1) which converts the pyrograms to numerical values for peak heights and calculates proportion of methylation at each base as a C/T ratio. All runs contained standard curves, which comprised a range of control methylated DNA (0%, 25%, 50%, 75%, and 100%) to allow standardized direct comparisons between different primer sets. For the standard curves a total of 300 ng of unmethylated (Qiagen) and hypermethylated DNA (Millipore, Billerica, MA, USA) were mixed to obtain the different ratios of DNA methylation and then bisulfite converted as described above.

In vitro Differentiation of human bone marrow derived HSCs

Human bone marrow-derived HSCs were isolated and differentiated *in vitro* as follows.

1. Isolation of human hematopoietic stem cells from bone marrow aspirate

Fresh bone marrow aspirate is obtained from donors. Bone fragments and cells are filtered through 40-μm cell strainer. Mononuclear cells from bone marrow aspirate are separated with histopaque-1077 (Sigma, 3000rpm, 30 min, room temperature). CD34+ Lineage- (CD4, CD8, CD11b, CD19, CD45R, CD161, GR.1, Ter119) progenitor cells or HSCs are isolated with flow cytometric cell sorting. If required, HSCs will be transfected with Lenti-Tet1 and selected by puromycin (5ug/ml). 5 × 103 cells (normal HSCs, or Tet1-overexpressing HSCs) are seeded in 10 mL of OP9 medium per 10-cm dish of 80%-90% confluent OP9 or OP9-DL1 cells. Add 5 ng/mL Flt-3L, 5ng/mL IL-3 and 1 ng/mL IL-7 for γδT cell differentiation. Add 5ng/mL GM-CSF, 5ng/mL IL-3 and 2ng/mL IL-2 for NKT cell differentiation.

2. In vitro differentiation

5 days later, disaggregate cells without the use of trypsin by pipetting the cells up and down until the OP9 cell monolayer is completely disrupted from the plate and

broken into small pieces. Filter cells through a 40-μm cell strainer. Wash the 10-cm dish with 6 mL of PBS, filter through the same cell strainer, and centrifuge at 400g (1500 rpm) for 5 min at 4°C. Resuspend the cells in 10 mL of OP9 medium (alpha MEM supplied with 20% FBS, 1% antibiotics) containing cytokines, and seed the cells onto 10-cm dishes of 80%-90% confluent fresh OP9 or OP9-DL1 cells. Measure NKT or γδT cell populations by FSCS 6 weeks after coculture.

3. IL-3 supplement

We bought bone marrow aspirate from All Cell Co Ltd. The samples were collected and delivered to our lab by overnight shipment on ice. We found that some HSCs isolated from the samples did not grow very well. To overcome the problem, we supplied 5ng/mL IL-3 in HSC culture medium. In the last culture, IL-3 supplement enhanced the proliferation of human HSCs. This observation is supported by previous studies (Bryder et al, Blood, 2000, 96, 1748). We did not have any evidence to show IL-3 supplement affects the differentiation of HSCs towards T lineages yet.

Statistical analysis

All data were shown as means ± sd. Statistical analyses were carried out with either GraphPad Prism (GraphPad Software) or SPSS v19 (IBM) software. Statistical significance was evaluated by using a one- or two-way analysis of variance (ANOVA) or an unpaired t-test. Significance was established for *P* values of at least <0.05.

Example 1. Hypercholesterolemia downregulates the expression of Tet1 in HSCs which functions as pivotal regulator in the differentiation from HSCs towards NKT and γδ T cells

Hypercholesterolemia (HC) increases the incidence and histopathologic severity of colorectal neoplasia by an HSC-autonomous mechanism.

Ten eleven translocation (Tet) family, including Tet1, Tet2 and Tet3, demethylate genomic DNA (Ito et al., Nature. 466, 1129-33 (2010); Ko et al., Nature. 468, 839-43 (2010); Ito et al., Science, 333, 1300 (2011).). Within the Tet family, Tet2 has been shown to have a critical role in regulating the self-renewal, proliferation and differentiation of HSCs (Ko et al., Nature. 468, 839-43 (2010); Ko et al., Proc Natl Acad Sci U S A. 108, 14566-71 (2011)), whereas the role of Tet1 in hematopoiesis is as yet unknown. In hematopoietic stem cells (HSC) of $ApoE^{-/-}$ mice

we found a significant downregulation of Tet1 (Figure 1a). Supplemental treatment with NAC restored the expression of Tet1 in HSCs from ApoE$^{-/-}$ mice (Figure 1b).

To test whether Tet1 plays a role in the differentiation of HSCs towards NKT and γδT cells, the expression of Tet1 in HSCs from WT and ApoE$^{-/-}$ mice was inhibited with shRNA (Figure 1c). The inhibition of Tet1 in HSCs from both WT and ApoE$^{-/-}$ mice greatly reduced their differentiation towards NKT and γδT cells both in vivo and in vitro (Figures 1d, 1e). In contrast, the overexpression of Tet1 in HSCs from WT or ApoE$^{-/-}$ mice resulted in 6-10 fold increase in the differentiation towards NKT cells and more than 20 fold increase in their differentiation towards γδT cells (Figures 1f, 1g, 1h, 1i, 1j).

Both *in vivo* and *in vitro*, NKT and γδT cells derived from Tet1-overexpressing HSCs had greater staining for HSA, a cell surface marker that decreases in expression with maturation (Figures 2i, 2j; Figure 3a, 3b). V1 subsets were decreased, while V2 and V6 subsets were significantly increased in γδT cells derived from Tet1-overexpressing HSCs (Figures 2c, 2d, 2e; Figures 3c, 3d, 3e). Interestingly, γδT cells derived from Tet1 overexpressing HSCs displayed greater expression of CCR6 and IL-17 (Figure 3f, 3g). Tet1 overexpression in HSCs also increased the differentiation towards CD8$^+$ T cells in in vitro differentiation assay (Figure 4). These results indicate that Tet1 is a pivotal determinant of the differentiation of HSCs towards NKT and γδT cells as well as their function.

In order to determine whether the overexpression of Tet1 in HSCs could restore the impaired immunosurveillance against colorectal neoplasia observed in hypercholesterolemic mice, we reconstituted the hematopoiesis of lethally irradiated WT recipient mice with WT HSCs, Tet1-overexpressing HSCs, ApoE$^{-/-}$ HSCs or Tet1-overexpressing ApoE$^{-/-}$ HSCs. Because Tet1-overexpressing HSCs were extremely quiescent and not able to fully reconstitute the hematopoiesis in lethally irradiated WT recipient mice, the transplantation with Tet1-overexpressing WT HSCs was supported with WT HSCs and the transplantation of Tet1-overexpressing ApoE$^{-/-}$ HSCs was supported with ApoE$^{-/-}$ HSCs at the ratio of 3:1. NKT and γδT cell populations in thymus of the recipient mice reconstituted with Tet1-overexpressing ApoE$^{-/-}$ HSCs was significantly greater than those in the recipient mice with ApoE$^{-/-}$ HSCs (Figure 5a, 5b). Similarly, the number of submucosal NKT and γδT cells were also significantly greater in the recipient mice reconstituted with Tet1-overexpressing

ApoE$^{-/-}$ HSCs than those in the recipient mice reconstituted with ApoE$^{-/-}$ HSCs (Figure 5c, 5d). In accordance with this increase in NKT and γδT cells, the average tumor number and histopathologic severity of colorectal neoplasia in the recipient mice reconstituted with Tet1-overexpressing ApoE$^{-/-}$ HSCs were significantly lower than those in the recipient mice with ApoE$^{-/-}$ HSC (Figures 5e, 5f). We also found that recipient mice reconstituted with Tet1-overexpressing WT HSCs had no carcinoma tumors (Figure 5f). These results indicate that transplantation with Tet1-overexpressing HSCs normalizes NKT and γδT cell population and also restored immunosurveillance against colorectal neoplasia.

Example 2. Tet1 epigenetically regulates the expression of genes critical in the differentiation toward NKT and γδT cells.

The differentiation and maturation of NKT and γδ T cells is regulated by the strict control of gene expression (Matsuda and Gapin, Curr Opin Immunol. 17(2), 122-30 (2005); Garbe and von Boehmer, Trends Immunol. 28(3), 124-31 (2007)). To identify the molecular mechanisms that underlie the decreased differentiation of NKT and γδ T cells in hypercholesterolemic mice, we screened the expression of genes critical to the differentiation of HSCs towards NKT and γδ T cells in in vitro differentiation assay (Table 1). We found lower expression of Fyn, Sox13, IL-15R, ITK and SH2D1a in the cells derived from ApoE$^{-/-}$ HSCs than those from WT HSCs. Overexpression of Tet1 in ApoE$^{-/-}$ HSCs restored the expression of these genes to a level even greater than those from WT HSCs. The overexpression of Tet1 also increased the expression of ETV5, BCL11b, EGR2, SLAMF1, ZBTB16, RELb, PHF1 and NFKb1 in the cells derived from both WT HSCs and ApoE$^{-/-}$ HSCs. These results indicate that Tet1 exerts a heretofore unrecognized significant influence on the network of transcription factors and other genes that regulate the differentiation towards NKT and γδ T cells.

TABLE 1

Genes related to iNKT cell differentiation	Genes related to γδ T cell differentiation
Interleukin-2 receptor β (IL-2Rb)	B-cell lymphoma/leukemia 11B (BCL11b)
Interleukin-15 receptor (IL-15R)	Early growth response protein 2 (EGR2)
E26 Transformation specific transcription factor 1 (Ets1)	Ets variant 5 (ETV5)
myeloid Elf-1-like factor (MEF)	inhibitor of DNA binding protein 2 (ID2)
Interferon regulatory factor 1 (IRF-1)	inhibitor of DNA binding protein 3 (ID3)
Fyn	interleukin-2-inducible T-cell kinase (ITK)
interleukin-2-inducible T-cell kinase (Itk)	Iterleukin 7 receptor (IL-7R)
Activator protein-1 (AP-1)	Interleukine-15 receptor (IL-15R)
T cell factor 1 (TCF-1)	PHD finger protein 1 (PHF1)
Nuclear factor κB p50 (NFκb)	SLAM-Associated Protein (SAP, SH2D1a)
RELb	*Sry*-related HMG box 13 (Sox13)
IκB kinase 2 (IKK2)	T cell factor 12 (TCF12)
Protein kinase C-θ (PKCθ)	Zinc finger and BTB domain-containing protein 16 (ZBTB16)
Signaling lymphocytic activation molecule F1 (SLAMF1)	
signaling lymphocytic activation molecule-associated protein (SAP)	
Krüppel-like factor 2 (KLF2)	
CCR9	

Thus, we have exposed human normal HSCs to oxidized-LDL and have shown a concentration-dependent impairment of their differentiation toward NKT and γδ T cells. In addition, exposure of human HSCs to oxidized-LDL also downregulates Tet1 as it does in mouse HSCs. Specifically, HC causes an oxidant-stress dependent downregulation of Tet1 in HSCs that reduces the expression of genes critical for γδ T cell and NKT cell differentiation. These effects reduce the concentration of γδ T cells and NKT cells in colon submucosa and at the early stages

of tumor development and thereby impair immunosurveillance against colorectal neoplasia. Overexpression of Tet1 in HSCs of HC mice restores their differentiation toward NKT and γδ T cells and reverses the increased incidence of colorectal neoplasia.

The results above showed that Tet1 is a crucial and essential determinant in the differentiation from HSCs towards NKT and γδ T cells as well as a pivotal role in the mechanism by which HC increases the incidence of colorectal neoplasia.. The overexpression of Tet1 in HSCs dramatically increased the differentiation of HSCs towards NKT and γδ T cells both *in vitro* and *in vivo*.

Example 3. Establishing *in vitro* and *in vivo* systems to enhance the differentiation of human HSCs towards NKT and γδ T cells.

Given that the Tet protein family is highly conserved in mammals, it was hypothesized that Tet1 also functions as a determinant in the differentiation of human HSCs to NKT and γδ T cells. To test this hypothesis, we will clone the full length human Tet1 or the catalytic domain of human Tet1 into lentiviral vectors. The lentiviral constructs yield among the best outcome to introduce DNA fragments or genes into human HSCs. Normal human HSCs and Tet1 overexpressing human HSCs will be selected and co-cultured with support cells which consistently express the critical molecule for T cell differentiation, Notch ligand Delta-like 1. The co-culture system is a reliable assay to study the *in vitro* differentiation of HSCs towards T cell lineages. It has been repeatedly used in numerous laboratories. The percentage of NKT and γδ T cells in the co-culture will be determined by flow cytometry 6-8 weeks following viral transduction. In the *in vivo* experiments, normal human HSCs or human HSCs overexpressing Tet1 will be injected intravenously (at a dose of $5x10^3$) into three month old lethally irradiated NOD-*scid IL2rγ*^{null} (NSG) humanized mice. The frequency of NKT and γδ T cells derived from human HSCs will be closely monitored at multiple time points after transplantation. In these experiments, we will measure the subsets of NKT and γδ T cells derived from normal human HSCs and Tet1 overexpressing human HSCs as well as the critical molecules and cytokines which are fundamental for the function of NKT and γδ T cells.

Example 4. Determining Tet1-dependent epigenetic regulation in the differentiation of human HSCs towards NKT and γδ T cells.

Current hematological research is raising the concern that even a highly enriched HSC fraction is heterogeneous in terms of lymphopoietic potential. Heritable epigenetic signatures of DNA, histone and chromosome conformation, appear to have a major role in the process (18, 19). Although the regulatory network governing the differentiation of HSCs towards NKT and γδ T cells has been extensively explored in the last decades, the epigenetic signature predisposing HSCs towards NKT and γδ T cell fate is yet unknown.

Tet-dependent DNA demethylation results in open chromatin structure and permits the transcription of target genes (Ko et al., Proc Natl Acad Sci U S A. 108, 14566-71 (2011); Wu and Zhang, Genes Dev. 25(23), 2436-52 (2011)). Pyrosequencing analysis showed that *Fyn, Sox13, IL-15R, EGR2 and SH2D1a* were highly methylated in the cells derived from $ApoE^{-/-}$ HSCs, supporting a Tet1-dependent down-regulation of the genes. The overexpression of Tet1 significantly decreased the methylation of most targeted genes in the cells derived from both WT and $ApoE^{-/-}$ HSCs, which correlates well with the high expression of the targeted genes in the cells derived from Tet1 overexpressing HSCs. These results indicate that Tet1-dependent demethylation regulates the expression of targeted genes that mediate HSC differentiation toward NKT and γδT cells.

However, we also found that the expression of BCL11b, RELb and PHF1 was increased in the cells derived from Tet1 overexpressing HSCs, but their methylation status was unchanged. In addition, although the methylation of *ETV5, EGR2, RELb* and *NFKB1* was significantly higher in the cells derived from $ApoE^{-/-}$ HSCs than those from WT HSCs, their expression was unchanged, indicating that the regulation of the genes responsible for NKT and γδT cell differentiation is more complex.

Recent studies indicate that Tet proteins may also participate in the regulation of histone modification via distinct pathways. The O-linked N-acetylglucosamine (O-GlaNAc) transferase OGT is an evolutionarily conserved enzyme that catalyzes O-linked protein glycosylation. Tet proteins were identified as stable partners of OGT in the nucleus (Vella et al., Mol Cell. 49(4), 645-56 (2013); Chen et al., Nature. 493(7433), 561-4 (2013); Shi et al., J Biol Chem. 288(29), 20776-84 (2013)). The interaction of Tet2 and Tet3 with OGT led to the GlcNAcylation of Host Cell Factor 1

and the integrity of H3K4 methyltransferase SET1/COMPASS complex, indicating that Tet proteins increase H3K4me3 that induces transcriptional activation (Deplus et al., EMBO J. 32(5), 645-55 (2013)). Although an early observation showed that the interaction between Tet1 and OGT was limited to embryonic stem cells (Bendelac et al., Annu Rev Immunol. 25, 297-336 (2007)), our immunoprecipitation studies indicate that OGT also has strong interactions with Tet1 in HSCs. In accordance with the decrease in Tet1 expression, the interaction with OGT was significantly reduced in HSCs isolated from hypercholesterolemic mice. The overexpression of Tet1 significantly increased the interaction of Tet1 and OGT, but did not influence the expression and interaction of Tet3 and OGT in the cells. H3K4me3 modification in all the genes except RELb and NFKB1 was increased after Tet1 overexpression, suggesting that by interacting with OGT Tet1 plays an important role in H3K4me3 modification in HSCs.

Our study showed that Tet1 increased the expression of genes critical in the differentiation of HSCs towards NKT and γδ T cells in mouse by demethylating the genes responsible for the differentiation from HSCs. We also have evidence that Tet1 also regulates the expression of genes by inducing histone protein modifications, primarily of H3K27me3 and H3K4me3. We harvest the T cells derived from normal human HSCs and Tet1 overexpressing human HSCs, and screen the expression of genes crucial in the differentiation of human HSCs towards NKT and γδ T cells. Then, we measure the DNA methylation status of these genes by using pyrosequencing, and measure H3K27me 3 and H3K4me3 as well as other histone modifications by using ChIP-PCR.

Example 5. The use of NKT and γδ T cells derived from Tet1 overexpressing HSCs in cancer immunotherapy.

We will apply two different approaches to demonstrate the use of NKT and γδ T cells derived from Tet1 overexpressing human HSCs. In the first approach, we will generate and purify NKT and γδ T cells in the *in vitro* co-culture system and inject them into NSG humanized mice which would have been implanted with human colorectal tumors. The cancer burden and the infiltration of NKT and γδ T cells derived from Tet1 overexpressing human HSCs into tumors will be determined at multiple time points. Furthermore, we will determine the capacity of these NKT and

γδ T cells to recognize and eliminate cancer cells *in vitro*. In the second approach, we will reconstitute the hematopoiesis of lethally irradiated NSG mice with normal human HSCs or Tet1 overexpressing human HSCs. Then, human colorectal cancer tissue will be implanted in the chimeric mice. The frequency of NKT and γδ T cells in peripheral blood will be closely monitored. The cancer burden and the infiltration of NKT and γδ T cells derived from Tet1 overexpressing human HSCs into tumors will be determined at multiple time points.

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OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of preparing a population of Natural Killer T cells (NKT) and/or γδ T cells, the method comprising:
obtaining a first population comprising hematopoietic stem cells (HSC);
engineering the HSC to overexpress Ten eleven translocation (Tet)1;
maintaining the Tet1-overexpressing HSC in culture under conditions and for a time sufficient for at least some of the HSC to differentiate into NKT and/or γδ T cells; and optionally purifying the NKT and/or γδ T cells,
thereby providing a population of NKT and/or γδ T cells.

2. A population of NKT and/or γδ T cells prepared by the method of claim 1.

3. A method of preparing a population of Natural Killer T cells (NKT) and/or γδ T cells for use in treating a subject who has cancer, the method comprising:
obtaining a first population comprising hematopoietic stem cells (HSC);
engineering the HSC to overexpress Ten eleven translocation (Tet)1;
maintaining the Tet1-overexpressing HSC in culture under conditions and for a time sufficient for at least some of the HSC to differentiate into NKT and/or γδ T cells; optionally purifying the NKT and/or γδ T cells.

4. The method of claim 3, wherein the first population of HSC is obtained from the subject who has cancer.

5. A population of NKT and/or γδ T cells prepared by the method of claims 3-4.

6. The population of NKT and/or γδ T cells of claim 5 for treating cancer in a subject.

7. A population of HSC engineered to overexpress exogenous Tet1.

8. The population of HSC of claim 6, wherein the HSC comprise a Tet1 gene operably linked to a regulatory region other than the endogenous Tet1 regulatory region.

9. The population of HSC of claims 6-7 for treating cancer in a subject.

10. The method of claims 3-6 and 9, wherein the cancer is colon cancer, ovarian cancer, prostate cancer, lymphoid malignancies, myeloma, renal cell carcinoma, breast cancer, or malignant glioma.

11. A method of preparing a population of Tet1-overexpressing HSC for increasing levels of NKT and/or γδ T cells in a subject, the method comprising:
obtaining a first population comprising hematopoietic stem cells (HSC);
engineering the HSC to overexpress Ten eleven translocation (Tet)1.

12. A method of preparing a population of Tet1-overexpressing HSC for increasing levels of NKT and/or γδ T cells in a subject, the method comprising:
obtaining a first population comprising hematopoietic stem cells (HSC);
engineering the HSC to overexpress Ten eleven translocation (Tet)1;
maintaining the Tet1-overexpressing HSC in culture under conditions and for a time sufficient for at least some of the HSC to differentiate into NKT and/or γδ T cells; and optionally purifying the NKT and/or γδ T cells.

13. The method of claims 11 or 12, wherein the first population of HSC is obtained from the subject.

14. The method of claims 11-13, wherein the subject has a tumor.

15. The method of claims 11-13, wherein the subject has carcinoma, sarcoma, myeloma, leukemia, or lymphoma.

16. The method of claims 11-13, wherein the subject has colon cancer, ovarian cancer, prostate cancer, lymphoid malignancies, myeloma, renal cell carcinoma, breast cancer, or malignant glioma.

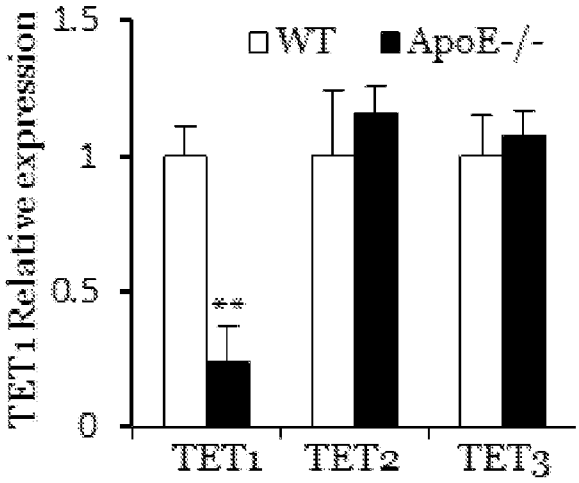

TET1 Relative expression
1.5
1
0.5
0
WT
ApoE-/-
**
TET1
TET2
TET3

FIG. 1A

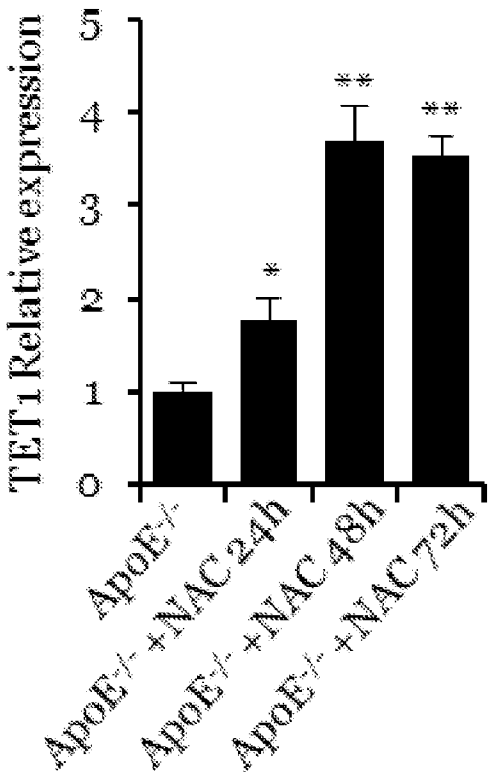

TET1 Relative expression
5
4
3
2
1
0
*
**
**
ApoE-/-
ApoE-/- +NAC 24h
ApoE-/- +NAC 48h
ApoE-/- +NAC 72h

FIG. 1B

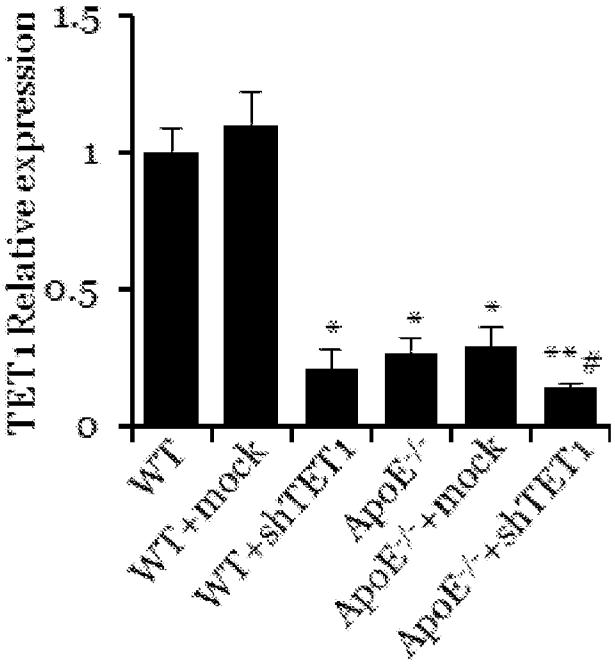

TET1 Relative expression
1.5
1
0.5
0
*
*
*
**#
WT
WT+mock
WT+shTET1
ApoE-/-
ApoE-/-+mock
ApoE-/-+shTET1

FIG. 1C

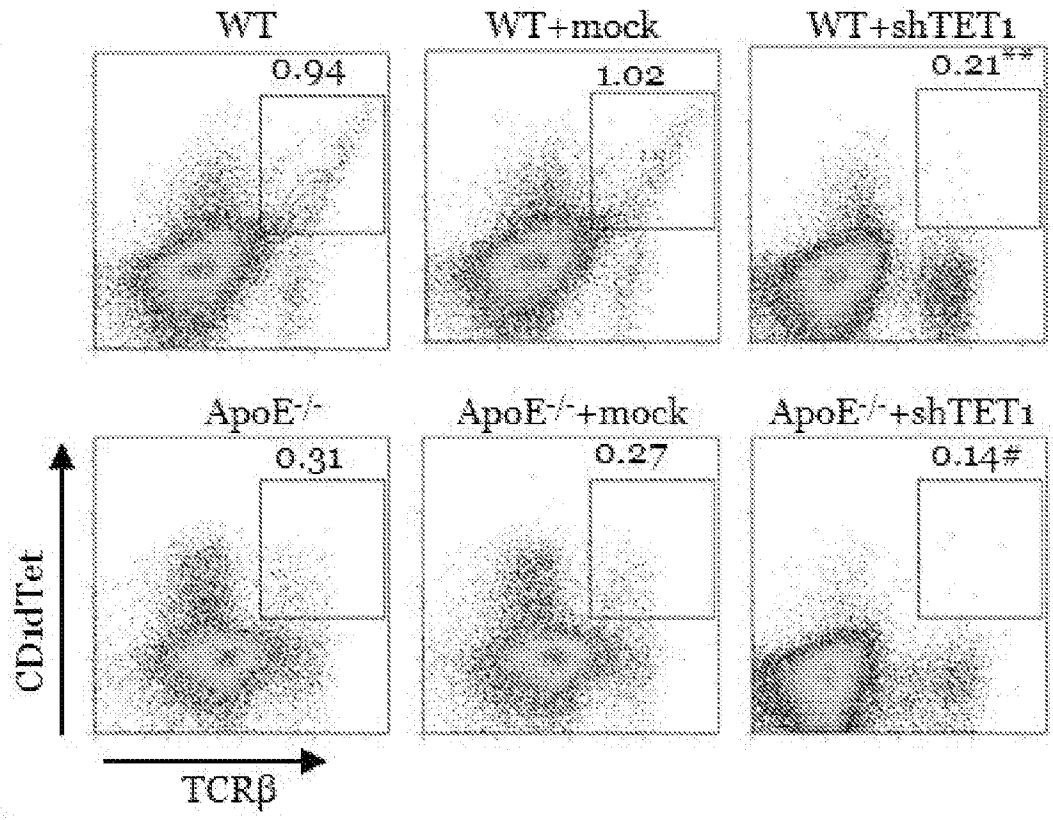

WT
0.94
WT+mock
1.02
WT+shTET1
0.21**
ApoE-/-
0.31
ApoE-/-+mock
0.27
ApoE-/-+shTET1
0.14#
CDudTet
TCRβ

FIG. 1D

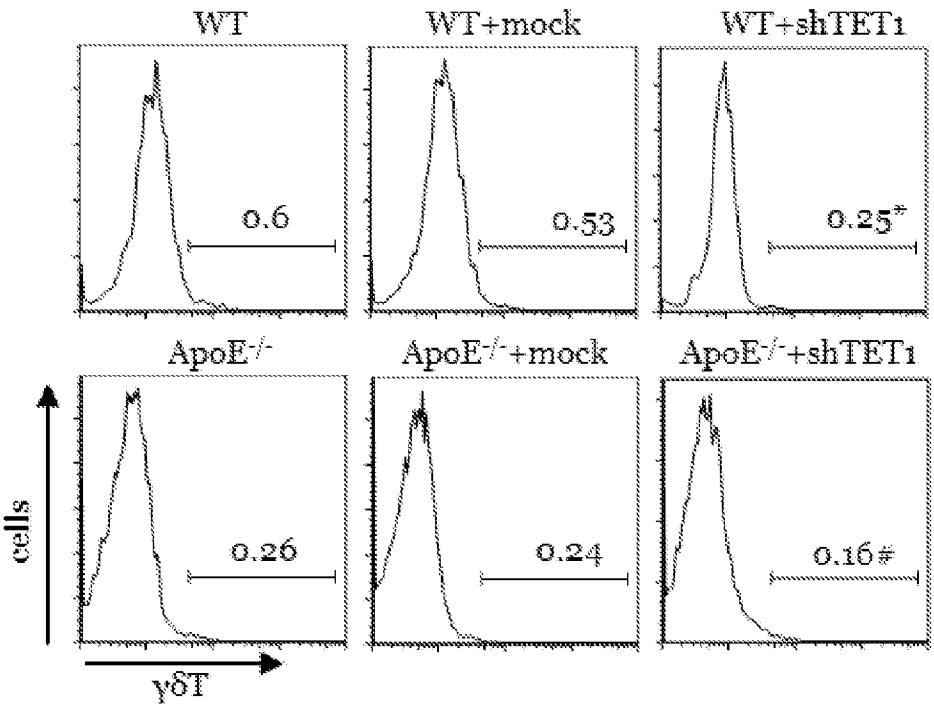

WT
WT+mock
WT+shTET1
0.6
0.53
0.25*
ApoE-/-
ApoE-/-+mock
ApoE-/-+shTET1
0.26
0.24
0.16#
cells
γδT

FIG. 1E

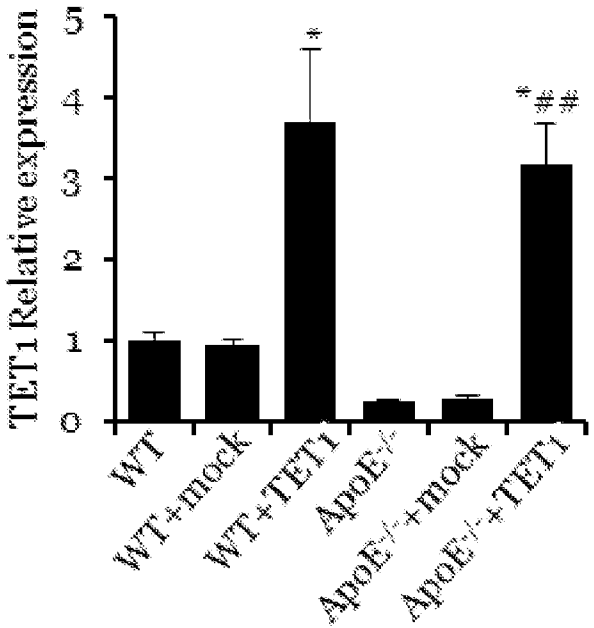

TET1 Relative expression
0
1
2
3
4
5
*
*##
WT
WT+mock
WT+TET1
ApoE-/-
ApoE-/-+mock
ApoE-/-+TET1

FIG. 1F

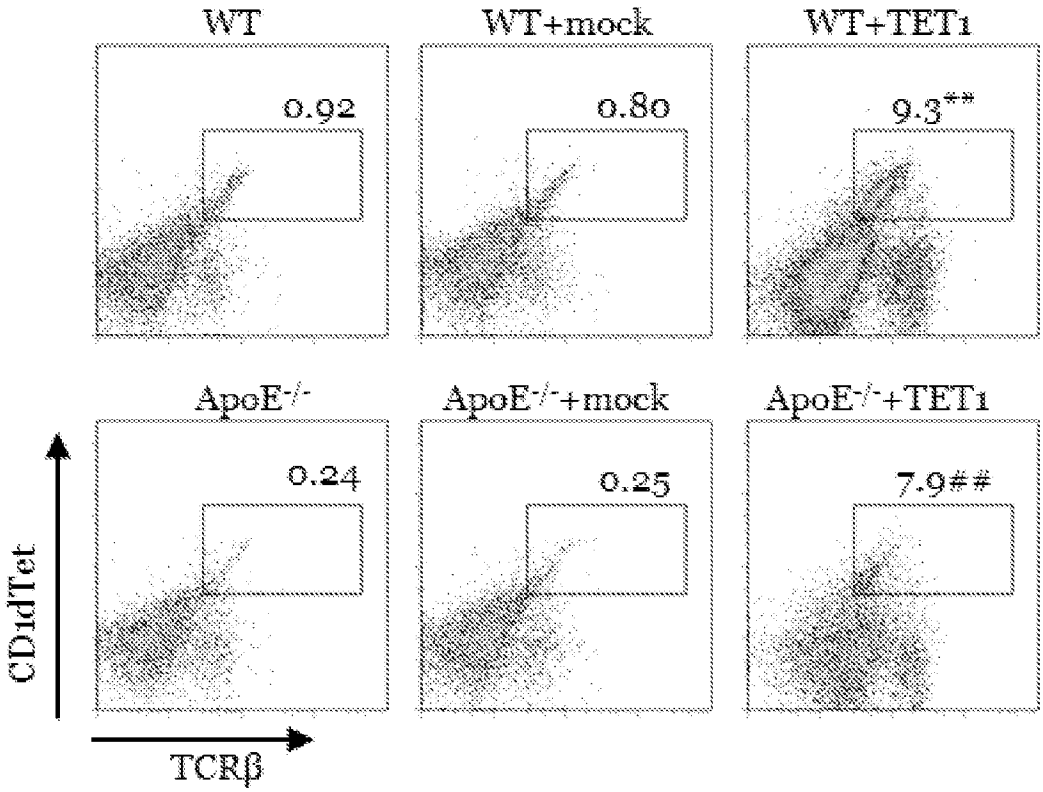

WT
WT+mock
WT+TET1
0.92
0.80
9.3**
ApoE-/-
ApoE-/-+mock
ApoE-/-+TET1
0.24
0.25
7.9##
CD1dTet
TCRβ

FIG. 1G

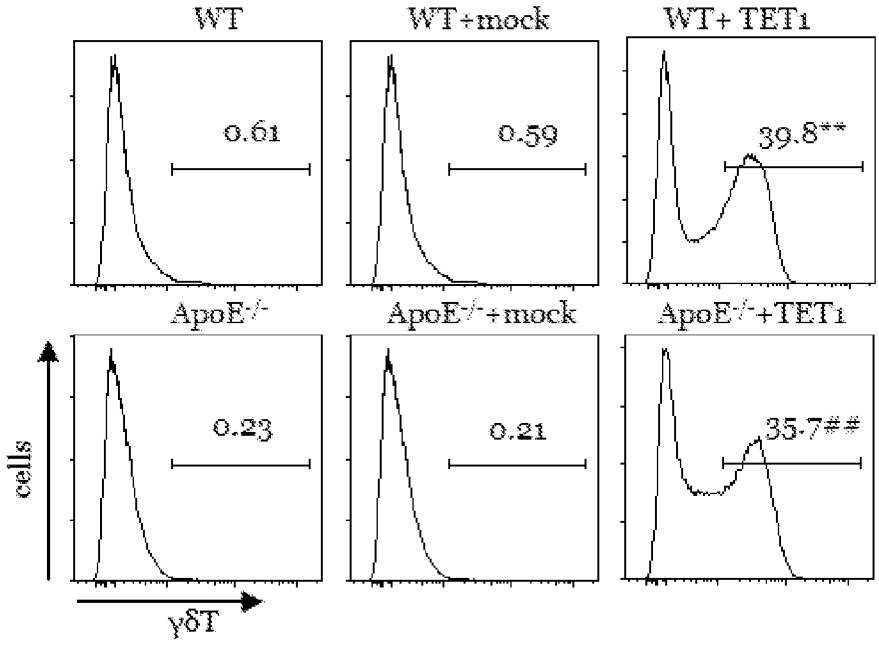

WT
WT+mock
WT+ TET1
0.61
0.59
39.8**
ApoE-/-
ApoE-/-+mock
ApoE-/-+TET1
0.23
0.21
35.7##
cells
γδT

FIG. 1H

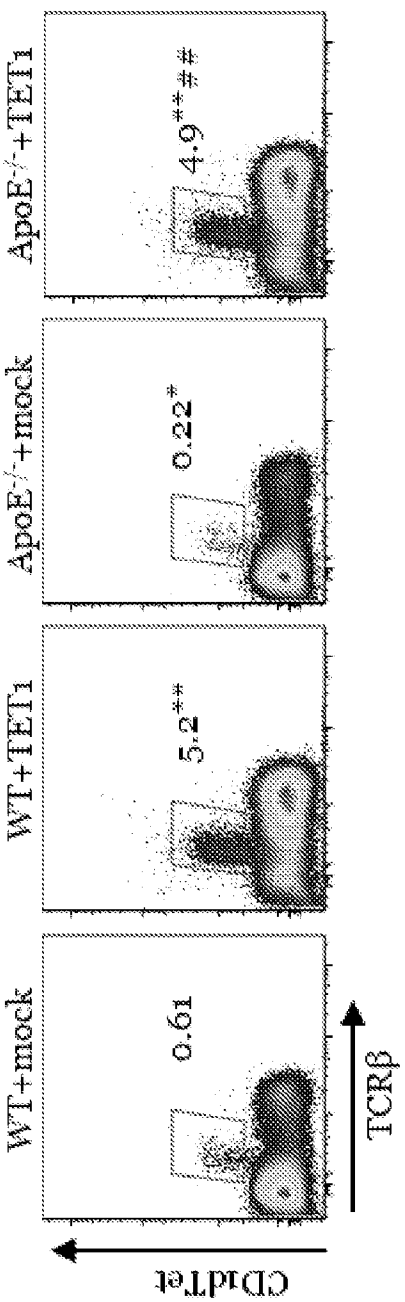

WT+mock
0.61
WT+TET1
5.2**
ApoE-/-+mock
0.22*
ApoE-/-+TET1
4.9**##
CD1dTet
TCRβ

FIG. 1I

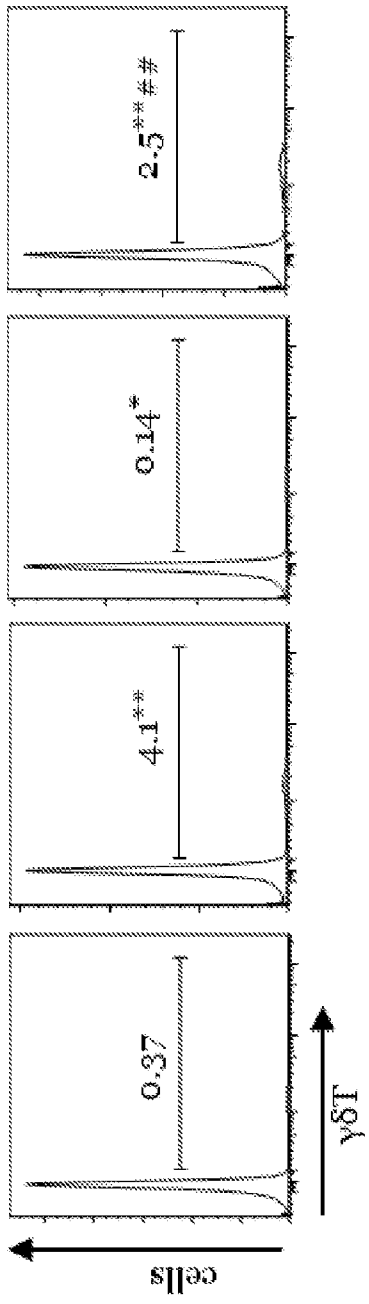

0.37
4.1**
0.14*
2.5**##
cells
γδT

FIG. 1J

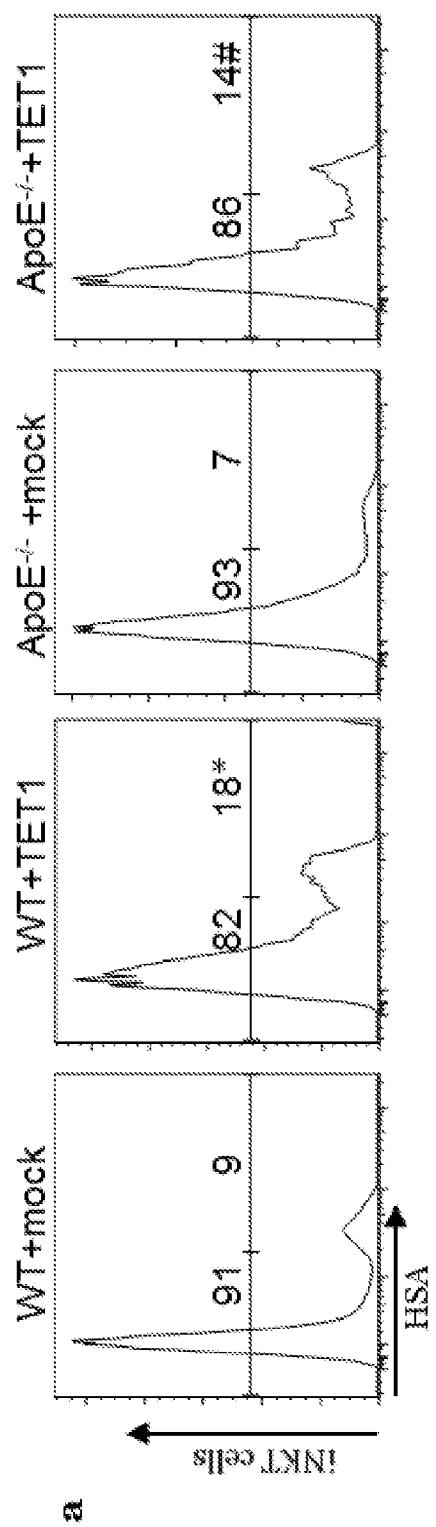

a
iNKT cells
HSA
WT+mock
91
9
WT+TET1
82
18*
ApoE-/- +mock
93
7
ApoE-/-+TET1
86
14#

FIG. 2A

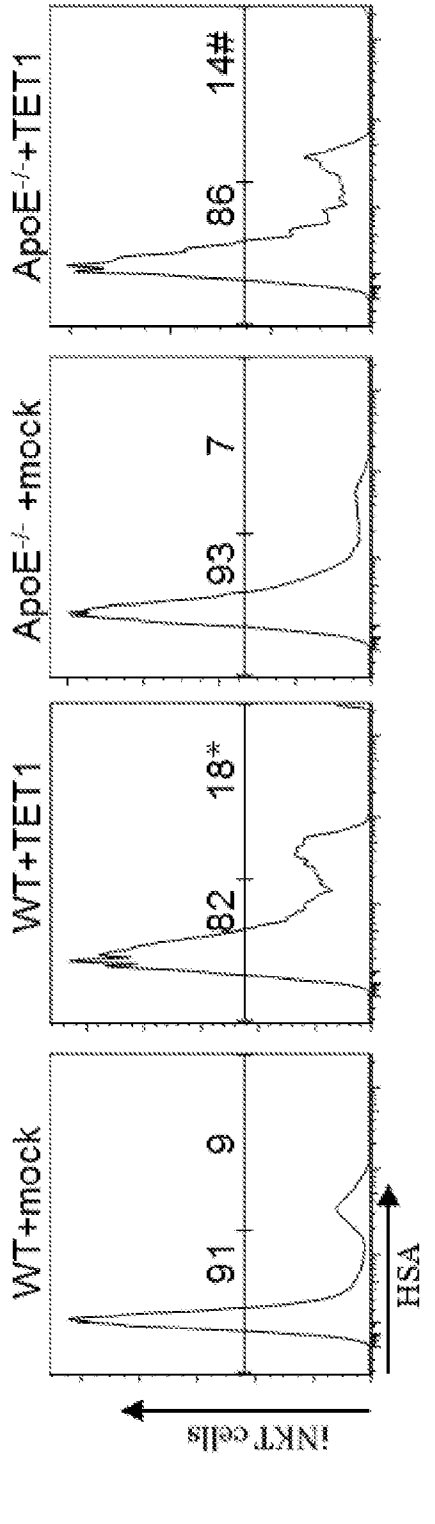

iNKT cells
HSA
WT+mock
91
9
WT+TET1
82
18*
ApoE-/- +mock
93
7
ApoE-/-+TET1
86
14#

FIG. 2B

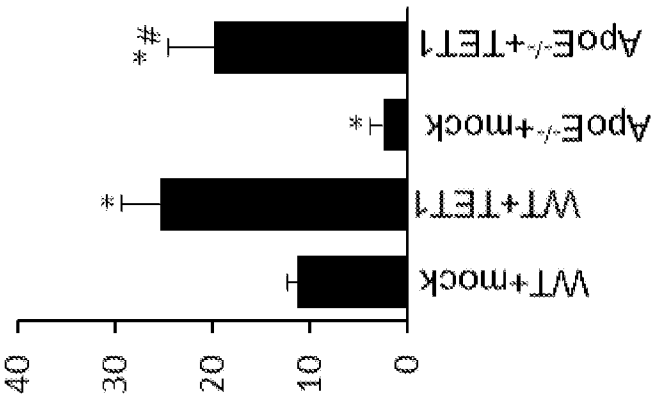


FIG. 2E

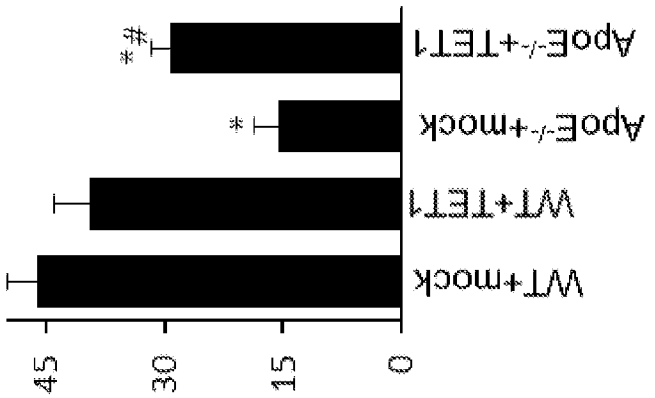


FIG. 2D

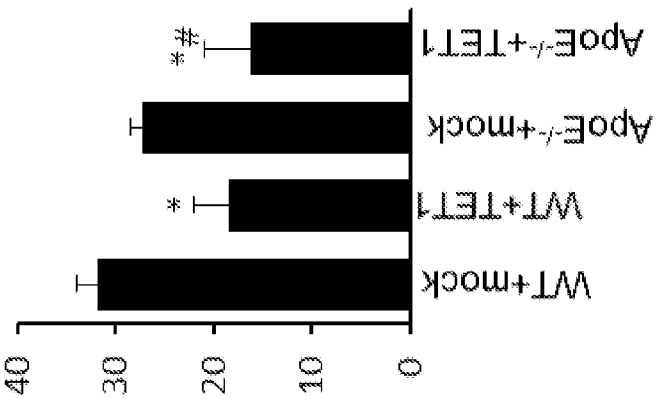


FIG. 2C

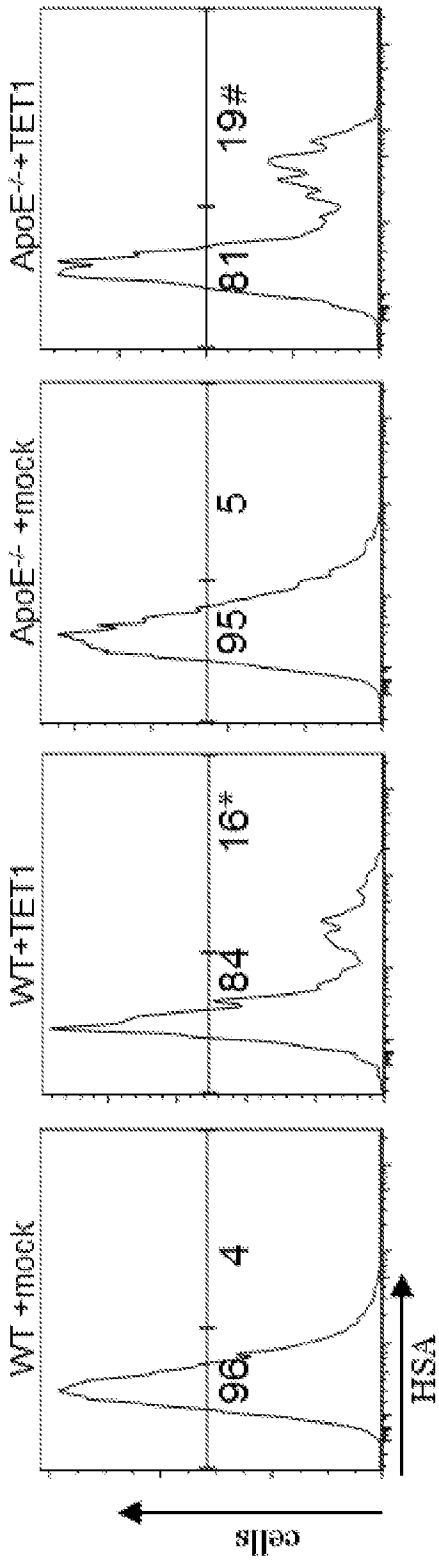

WT +mock
WT+TET1
ApoE-/- +mock
ApoE-/-+TET1
96
4
84
16*
95
5
81
19#
cells
HSA

FIG. 3A

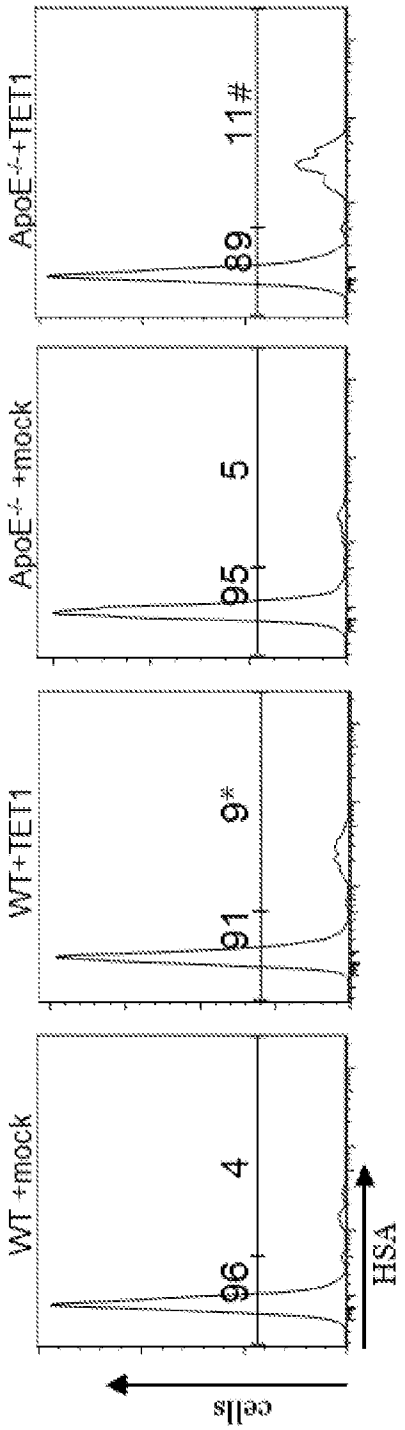

WT +mock
WT+TET1
ApoE-/- +mock
ApoE-/-+TET1
96
4
91
9*
95
5
89
11#
cells
HSA

FIG. 3B

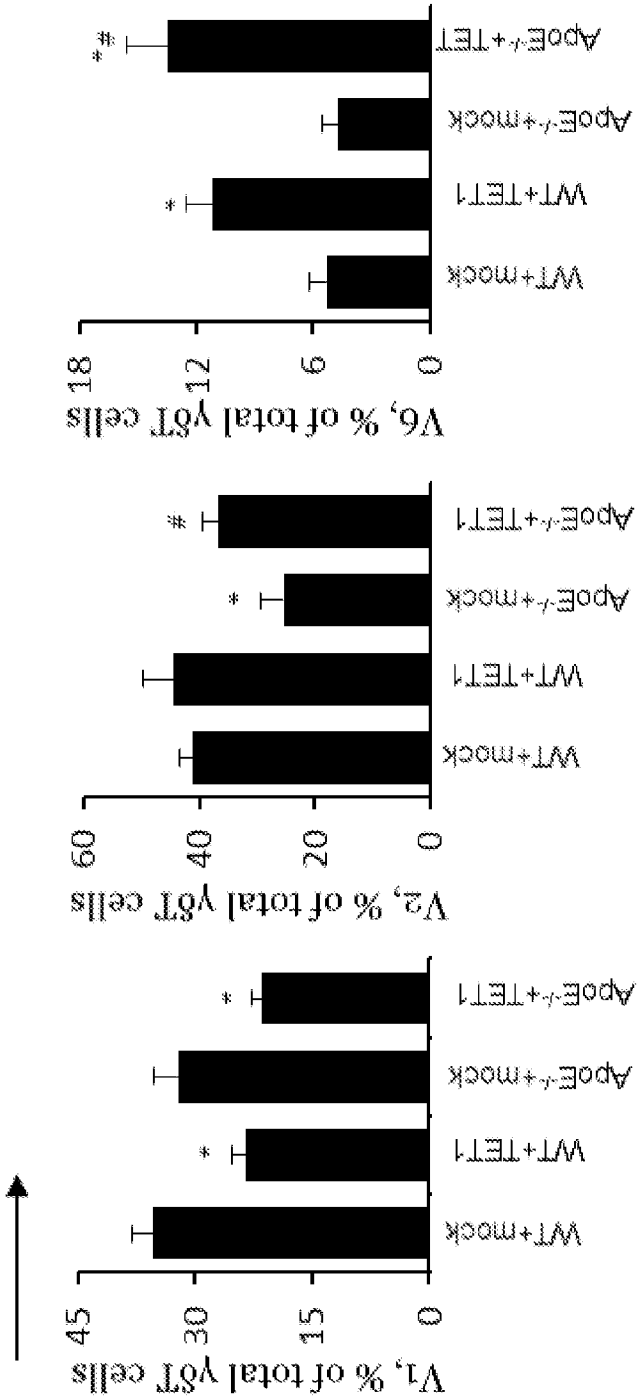


FIG. 3C

FIG. 3D

FIG. 3E

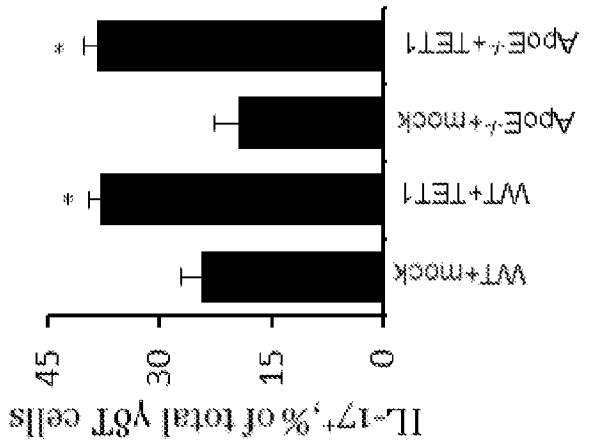

IL-17+, % of total γδT cells
45
30
15
0
WT+mock
WT+TET1
ApoE-/-+mock
ApoE-/-+TET1
*
*

FIG. 3G

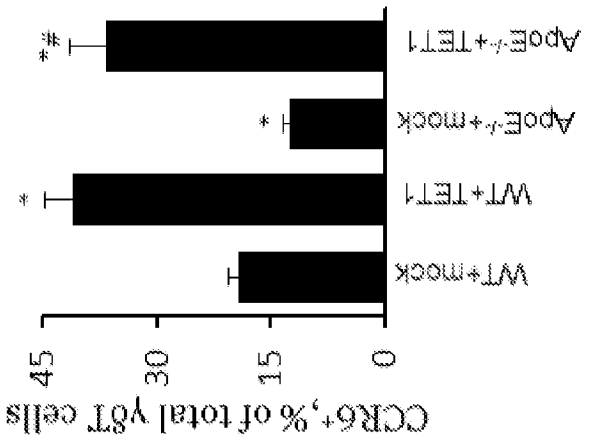

CCR6+, % of total γδT cells
45
30
15
0
WT+mock
WT+TET1
ApoE-/-+mock
ApoE-/-+TET1
*
*
**

FIG. 3F

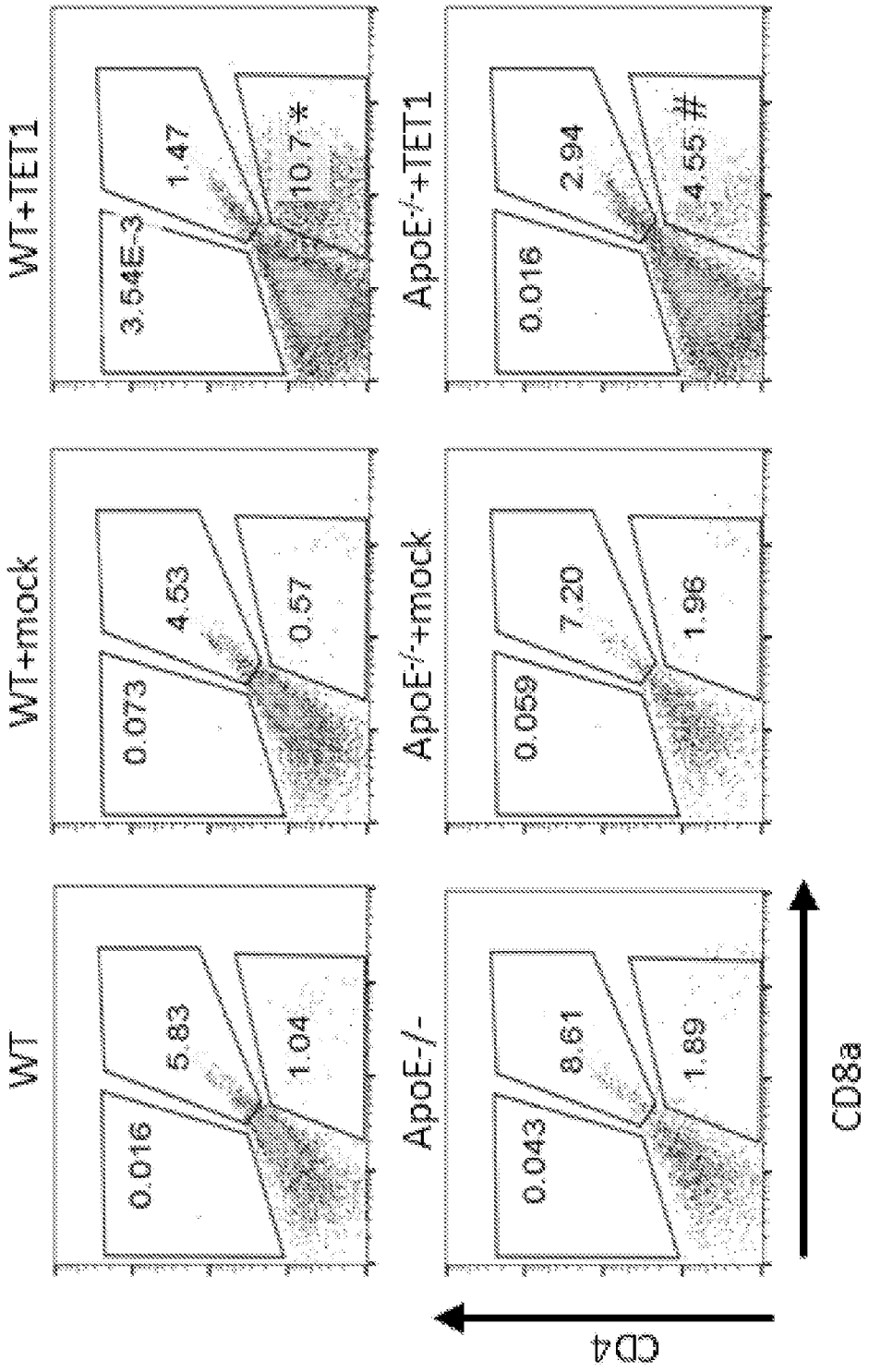

WT
0.016
5.83
1.04
WT+mock
0.073
4.53
0.57
WT+TET1
3.54E-3
1.47
10.7 *
ApoE-/-
0.043
8.61
1.89
ApoE-/-+mock
0.059
7.20
1.96
ApoE-/-+TET1
0.016
2.94
4.55 #
CD8a
CD4

FIG. 4

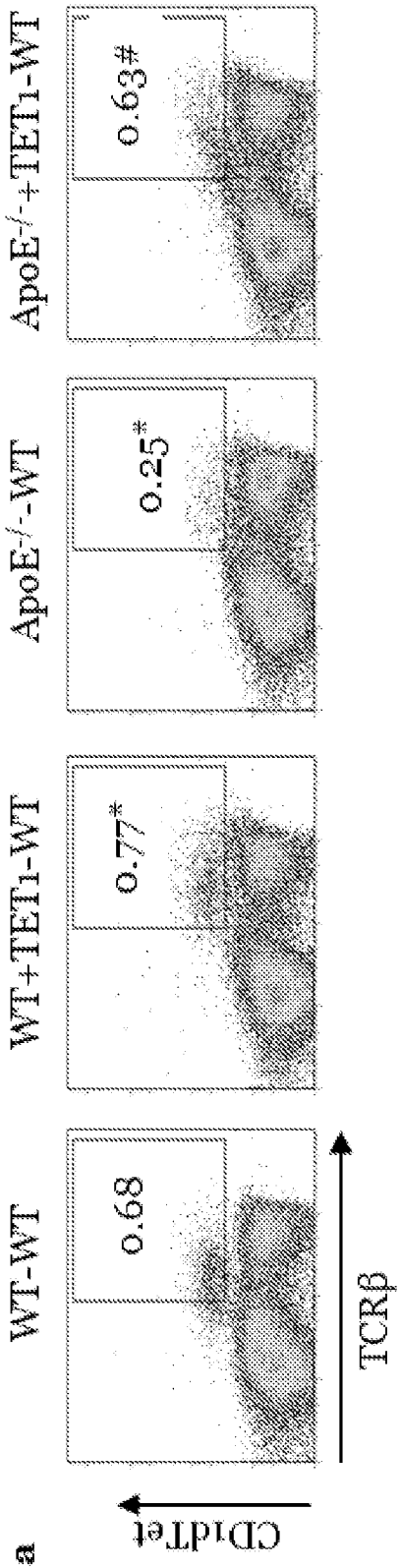

a
CD1dTet
TCRβ
WT-WT
0.68
WT+TET1-WT
0.77*
ApoE-/-WT
0.25*
ApoE-/-+TET1-WT
0.63#

FIG. 5A

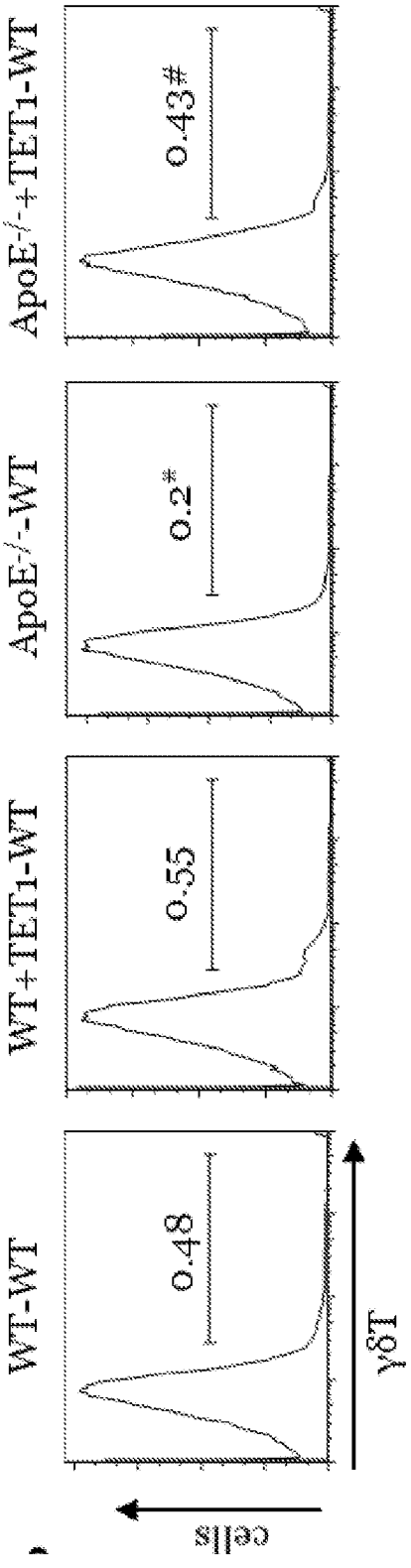

b
cells
γδT
WT-WT
0.48
WT+TET1-WT
0.55
ApoE-/-WT
0.2*
ApoE-/-+TET1-WT
0.43#

FIG. 5B

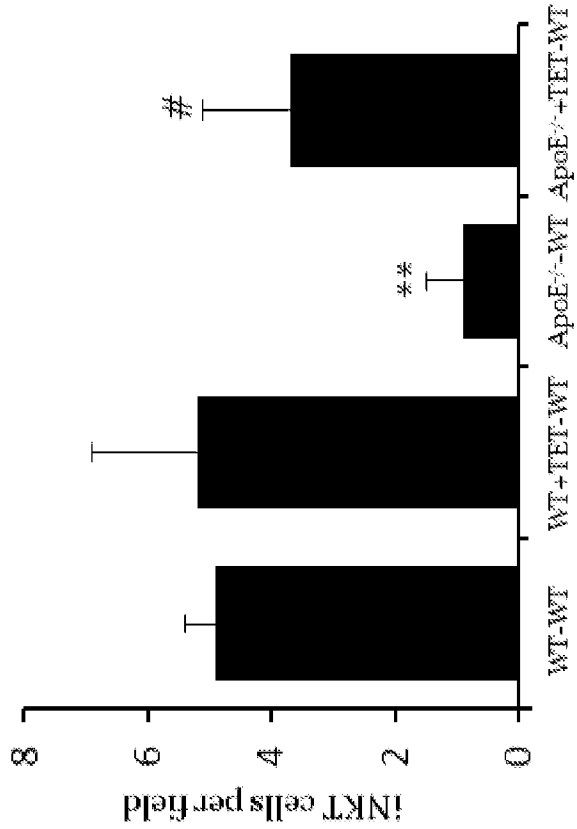


FIG. 5C

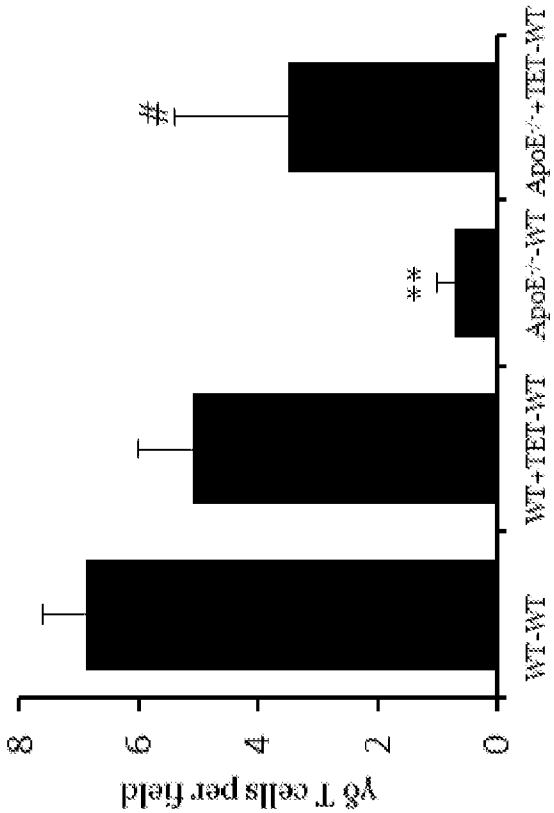


FIG. 5D

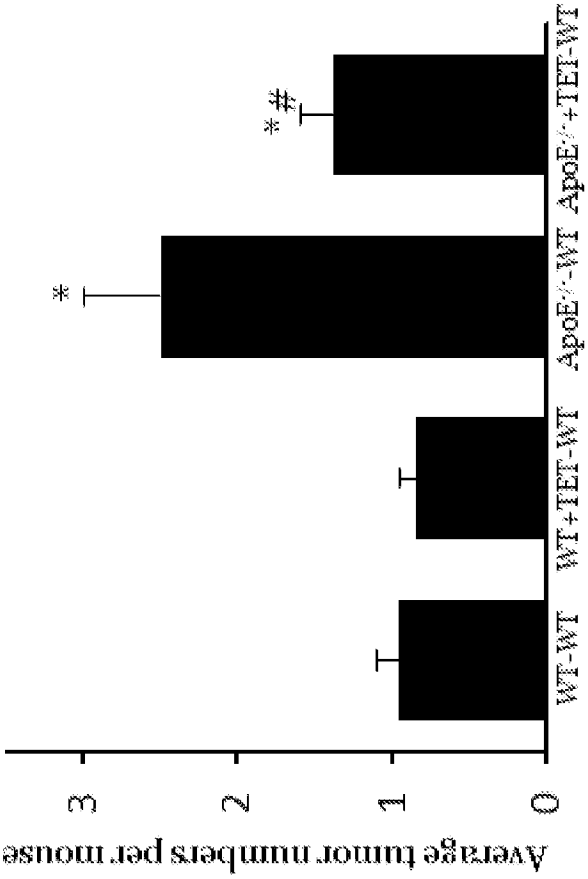

Average tumor numbers per mouse
0
1
2
3
WT-WT
WT+TET-WT
ApoE-/--WT
ApoE-/-+TET-WT
*
*#

FIG. 5E

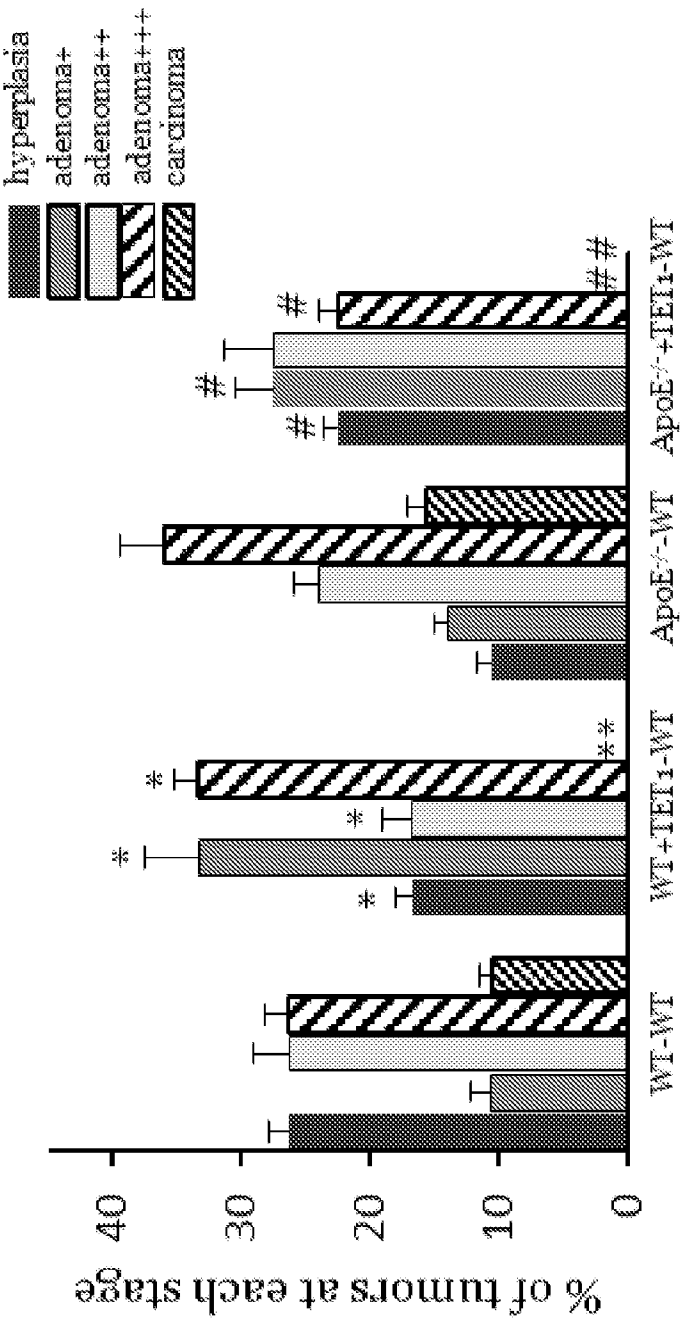

hyperplasia
adenoma+
adenoma++
adenoma+++
carcinoma
% of tumors at each stage
0
10
20
30
40
WT-WT
WT+TET1-WT
ApoE-/--WT
ApoE-/-+TET1-WT

FIG. 5F

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/14477

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 5/0783, C12N 5/0789 (2016.01)
CPC - A61K35/28, C12N5/0647, C12N5/0696
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)
IPC(8) - C12N 5/0783, C12N 5/0789 (2016.01)
CPC - A61K35/28, C12N5/0647, C12N5/0696

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/377, 514/44, 435/455
Keyword Search, search terms below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar. Natural killer T cells, NKT, gamma-delta T cells, hematopoeitic stem cell, HSC, ten eleven translocation, TET1, overexpress, methylation, demethylation, immunotherapy, expansion, differentiation, transcription factor,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---------- Y	US 2013/0323220 A1 (THE GENERAL HOSPITAL CORPORATION) 05 December 2013 (05.12.2013) para [0004], [0100], [0101], [0102], [0124], [0125], [0126]	7-8 ---------- 1-4, 11-13
Y	"A Role for DNA Hypomethylation and Histone Acetylation in Maintaining Allele-Specific Expression of Mouse NKG2A in Developing and Mature NK Cells" (Rogers et al.) J Immunol 2006; 177:414-421, abstract, pg 418, col 2, para 2	1-4, 11-13
Y	US 2011/0236362 A1 (WATARAI et al.) 29 September 2011 (29.09.2011) para [0106], [0107], [0110],	3-4

☐ Further documents are listed in the continuation of Box C. ☐

* 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	Date of mailing of the international search report
17 March 2016 (17.03.2016)	22 APR 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/14477

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-6, 9-10, 14-16
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.