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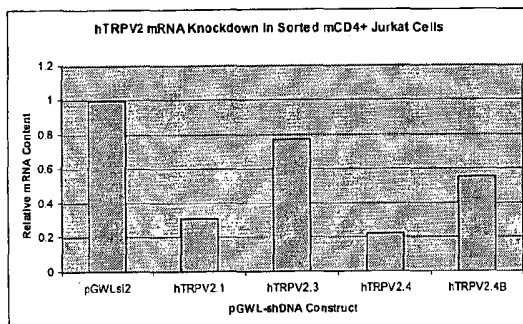
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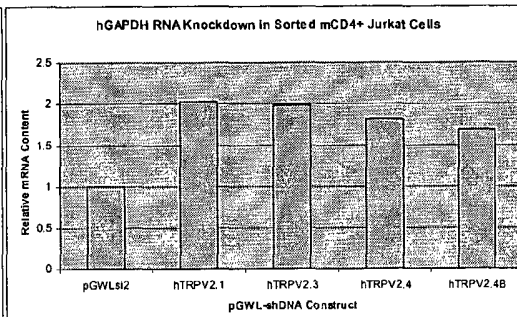
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(54) Title: METHODS FOR IDENTIFYING T CELL ACTIVATION-MODULATING COMPOUNDS

Probe: hTRPV2



Probe: hGAPDH



(57) Abstract: This invention provides novel methods for identifying compounds that modulate T cell activation and function. The modulators are identified by screening test compounds for ability to modulate TRPV2 mediated calcium influx into cells (e.g., a T cell). Such TRPV2 modulators can be further examined for their activity in modulating T cell activation (e.g., T cell differentiation or proliferation). Pharmaceutical compositions comprising these T cell modulators can be administered to a subject to modulate T cell immune responses, to suppress inflammations, and to treat disease conditions such as autoimmune diseases, graft rejection, cancer or allergies.

METHODS FOR IDENTIFYING T CELL ACTIVATION-MODULATING COMPOUNDS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority to U.S. Provisional Patent Application Serial No. 60/635,997 (filed December 14, 2004), the disclosure of which is incorporated herein by reference in its entirety and for all purposes.

FIELD OF THE INVENTION

[0002] The present invention generally relates to methods for identifying modulators of transient receptor potential cation channel, subfamily V, member 2 (TRPV2 or VRL-1) and therapeutic applications of such modulators. More particularly, the invention pertains to novel TRPV2 inhibitors that modulate T lymphocyte activation, and to methods of using such modulators to treat diseases and conditions mediated by abnormal T cell activities.

BACKGROUND OF THE INVENTION

[0003] T cell activation is initiated via engagement of the T cell receptor (TCR). Stimulation of the TCR results in a transient release of stored Ca^{2+} and a subsequent transient increase in the cytoplasmic Ca^{2+} concentration. The transient depletion of intracellular Ca^{2+} stores triggers the opening of Ca^{2+} Release Activated Ca^{2+} Channels (CRAC) in the plasma membrane, allowing for a sustained capacitative Ca^{2+} influx. Calcium influx from extracellular sources through CRAC in the cell membrane is essential for T cell activation, proliferation, and activation of effector functions (see, e.g., Lewis, R.S., Annu. Rev. Immunol. 19:497–521, 2001; and Feske et al., Biochem Biophys Res Commun 311: 1117-32, 2003). T cell activation can be impaired by inhibiting calcium influx. For example, it was shown that TGF- β limits T cell differentiation along both the Th1 and Th2 lineages by inhibiting the Itk kinase and consequently calcium influx (Chen et

al., J Exp Med. 197:1689-99, 2003). Human patients with impaired Ca^{2+} -release response show severe immunodeficiency due to complete loss of peripheral T cell function, even though T cell development is normal (see, e.g., Feske et al., Immunobiology 202: 134-50, 2000; and Feske et al., Nat Immunol 2: 316-24, 2001).

[0004] While normal T cells are an integral part of mammalian immune responses, in some instances it is desirable to inhibit undesirable immune responses such as undesirable proliferation of T cells. For instance, autoimmune diseases are characterized as an immune reaction against "self" antigens. Autoimmune diseases include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), type I diabetes and multiple sclerosis (MS). T cell responses have also been implicated in graft rejection, allergy, asthma, dermatitis, psoriasis and graft versus host disease (GVHD). Thus, treatment directed to inhibition of T cell activation would be greatly desired to treat such undesired immune responses.

[0005] There is a need for new compounds and methods for inhibiting T cell immune responses and for treating the above-noted diseases and conditions. By providing novel methods and compositions for modulating T cell activation and function, the instant invention addresses this and other needs.

SUMMARY OF THE INVENTION

[0006] In one aspect, the present invention provides methods for identifying agents that modulate T cell activation and function. The methods entail assaying test compounds for ability to modulate calcium influx into a cell mediated by transient receptor potential cation channel, subfamily V, member 2 (TRPV2). In some methods, the cell employed is a T lymphocyte. The T lymphocyte can be selected from the group consisting of thymus cells, $\text{CD4}^+\text{CD8}^+$ T cells, CD4^+ T cells, CD8^+ T cells, Th1, Th2, Treg, $\text{CD4}^+\text{CD25}^+$ T cells, $\text{T}_{\text{CT}}1$, $\text{T}_{\text{CT}}2$, Tr1, Ts, $\text{T}\gamma\delta$, and natural killer T cell (NKT). The T lymphocyte employed in the screening can also be a cultured T cell line, e.g., Jurkat cell. In some other methods, the cell employed is a cell transiently expressing TRPV2, e.g., a HEK293 cell or a CHO cell. The cell can be transfected with a cDNA encoding human or murine TRPV2. In some methods, calcium influx into the cell is monitored by measuring intracellular calcium level

in the cell. Some of the methods are directed to identifying agents that inhibit TRPV2-mediated calcium influx into the cell.

[0007] In a related aspect, the invention provides methods for identifying agents that modulates T lymphocyte activation and function. These methods involve assaying a biological activity of TRPV2 in the presence of test compounds to identify a modulating agent that modulates the biological activity of the TRPV2. The methods can further include testing the identified modulating agent for ability to modulate a T cell activation-related activity.

[0008] In some of these methods, the TRPV2 molecule employed in the screening is human or murine TRPV2. The assayed biological activity of TRPV2 can be its phosphorylation or induction of whole cell current. In some methods, TRPV2 phosphorylation monitored in the screening is its phosphorylation by cAMP-dependent kinase (PKA). In some other methods, induction of whole cell current by TRPV2 is monitored by measuring intracellular calcium level in a cell transiently expressing TRPV2. The cell employed can be a HEK293 cell or a CHO cell transiently transfected with a cDNA encoding human or murine TRPV2.

[0009] In some methods, the T cell activation-related activity is calcium influx into a cultured T cell, e.g., a Jurkat cell, and calcium influx into the T cell is monitored by measuring intracellular calcium levels in the cell. In some other methods, the T cell activation-related activity is proliferation or differentiation of a T cell, e.g., a CD4⁺ T cell or a CD8⁺ T cell. Some of the methods are directed to identifying agents that inhibit the T cell activation-related activity.

[0010] In another aspect, the present invention provides methods for suppressing an undesired T lymphocyte response in a subject. These methods entail administering to the subject an effective amount of a compound which inhibits TRPV2-mediated calcium influx into a T cell. Some of the compounds inhibit calcium influx into a cell mediated by human TRPV2. In some of the methods, the subject treated suffers from an autoimmune disease, a graft rejection, or a cancer. Examples of autoimmune diseases that can be treated include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), or multiple sclerosis (MS).

[0011] A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figure 1 shows knockdown of TRPV2 mRNA expression in Jurkat cells via RNAi. Jurkat cells were nucleofected with expression constructs harboring no shDNA (pGWLsi2) or either one of 4 shDNAs targeting different regions of the human TRPV2 mRNA (hTRPV2.1, hTRPV2.3, hTRPV2.4, hTRPV2.4B) along with a plasmid driving expression of the extracellular domain of murine CD4 (mCD4). 3 days post nucleofection, total RNA was prepared from FACS-sorted mCD4⁺ cells and analyzed for its content of hTRPV2 (left panel) or hGAPDH (right panel) mRNA via TaqMan RT-PCR. Results are shown normalized to the respective expression levels in vector nucleofected control cells. Two independently conducted experiments yielded comparable results.

[0013] Figure 2 shows inhibition of OKT3-induced Ca²⁺ mobilization in Jurkat cell clones expressing a dominant negative human TRPV2 mutant.

DETAILED DESCRIPTION

I. Overview

[0014] The invention is predicated in part on the discovery by the present inventors that transient receptor potential cation channel, subfamily V, member 2 (TRPV2 or VRL-2) plays an important role in T cell activation. As detailed in the Examples below, the present inventors identified the Ca²⁺ channel TRPV2 as one molecular component of calcium channels mediating sustained calcium influx in T cells, and that knockdown of TRPV2 impairs TCR and calcium signaling. Specifically, it was observed that Jurkat cells nucleofected with shDNAs against human TRPV2 showed defects in T cell receptor or Thapsigargin-induced calcium mobilization, with predominant effects on the sustained phase of calcium influx, which is known to be CRACC mediated. In addition, it was observed that a dominant negative TRIA-hTRPV2 can inhibit endogenous channels

mediating Ca²⁺ influx. These data indicate that TRPV2 could play an important role in regulating the Ca²⁺-release machinery and hence in T cell activation.

[0015] In accordance with these discoveries, the present invention provides methods for screening novel agents that modulate T cell activation and function. Test compounds are first examined for their ability to modulate a biological activity of a TRPV2 molecule, e.g., its expression or its ion channel activity. The agents thus identified can then be further tested for ability to modulate T cell activation. Various TRPV2 molecules can be employed in the screening assays. For example, TRPV2 from human, rat or mouse can be used to screen the modulators. In preferred embodiments, a human TRPV2 is used.

[0016] The methods of the present invention also find therapeutic applications. Pharmacological inhibition of TRPV2 provides a novel approach for treating various medical conditions such as autoimmune diseases or other conditions with undesired T cell responses. By inhibiting T cell activation (e.g., proliferation or differentiation), the TRPV2 modulators of the present invention are useful for treating a number of diseases in human and non-human subjects. These include transplant rejection or other inflammatory disorders mediated by T cells, e.g., autoimmune disease (e.g. Rheumatoid Arthritis, SLE, Diabetes, Neurodegenerative Disorders), Psoriasis, COPD, Allergy (Asthma, Rhinitis, Dermatitis) and others. Typically, the approach entails administering to a subject a TRPV2 modulator (e.g., an antagonist) that can be identified in accordance with the present invention.

[0017] The following sections provide guidance for making and using the compositions of the invention, and for carrying out the methods of the invention.

II. Definitions

[0018] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention pertains. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY (2d ed. 1994); THE CAMBRIDGE DICTIONARY OF SCIENCE AND TECHNOLOGY (Walker ed., 1988); and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY (1991). In addition, the following definitions are provided to assist the reader in the practice of the invention.

[0019] The term "agent" or "test agent" includes any substance, molecule, element, compound, entity, or a combination thereof. It includes, but is not limited to, e.g., protein, polypeptide, small organic molecule, polysaccharide, polynucleotide, and the like. It can be a natural product, a synthetic compound, or a chemical compound, or a combination of two or more substances. Unless otherwise specified, the terms "agent", "substance", and "compound" can be used interchangeably.

[0020] The term "analog" is used herein to refer to a molecule that structurally resembles a reference molecule but which has been modified in a targeted and controlled manner, by replacing a specific substituent of the reference molecule with an alternate substituent. Compared to the reference molecule, an analog would be expected, by one skilled in the art, to exhibit the same, similar, or improved utility. Synthesis and screening of analogs, to identify variants of known compounds having improved traits (such as higher binding affinity for a target molecule) is an approach that is well known in pharmaceutical chemistry.

[0021] As used herein, "contacting" has its normal meaning and refers to combining two or more agents (e.g., polypeptides or small molecule compounds) or combining agents and cells (e.g., a polypeptide and a cell). Contacting can occur in vitro, e.g., combining two or more agents or combining a test agent and a cell or a cell lysate in a test tube or other container. Contacting can also occur in a cell or in situ, e.g., contacting two polypeptides in a cell by coexpression in the cell of recombinant polynucleotides encoding the two polypeptides, or in a cell lysate.

[0022] A "heterologous sequence" or a "heterologous nucleic acid," as used herein, is one that originates from a source foreign to the particular host cell, or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that, although being endogenous to the particular host cell, has been modified. Modification of the heterologous sequence can occur, e.g., by treating the DNA with a restriction enzyme to generate a DNA fragment that is capable of being operably linked to the promoter. Techniques such as site-directed mutagenesis are also useful for modifying a heterologous nucleic acid.

[0023] The term "homologous" when referring to proteins and/or protein sequences indicates that they are derived, naturally or artificially, from a common ancestral protein or

protein sequence. Similarly, nucleic acids and/or nucleic acid sequences are homologous when they are derived, naturally or artificially, from a common ancestral nucleic acid or nucleic acid sequence. Homology is generally inferred from sequence similarity between two or more nucleic acids or proteins (or sequences thereof). The precise percentage of similarity between sequences that is useful in establishing homology varies with the nucleic acid and protein at issue, but as little as 25% sequence similarity is routinely used to establish homology. Higher levels of sequence similarity, e.g., 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or more can also be used to establish homology. Methods for determining sequence similarity percentages (e.g., BLASTP and BLASTN using default parameters) are described herein or well known and readily available in the art.

[0024] A "host cell," as used herein, refers to a prokaryotic or eukaryotic cell that contains heterologous DNA that has been introduced into the cell by any means, e.g., electroporation, calcium phosphate precipitation, microinjection, transformation, viral infection, and/or the like.

[0025] The terms "identical" or "sequence identity" in the context of two nucleic acid sequences or amino acid sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified comparison window. A "comparison window", as used herein, refers to a segment of at least about 20 contiguous positions, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are aligned optimally. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2:482; by the alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443; by the search for similarity method of Pearson and Lipman (1988) Proc. Nat. Acad. Sci U.S.A. 85:2444; by computerized implementations of these algorithms (including, but not limited to CLUSTAL in the PC/Gene program by Intelligentics, Mountain View, CA; and GAP, BESTFIT, BLAST, FASTA, or TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wis., U.S.A.). The CLUSTAL program is well described by Higgins and Sharp (1988) Gene 73:237-244; Higgins and Sharp (1989) CABIOS 5:151-153; Corpet et al.

(1988) *Nucleic Acids Res.* 16:10881-10890; Huang et al (1992) *Computer Applications in the Biosciences* 8:155-165; and Pearson et al. (1994) *Methods in Molecular Biology* 24:307-331. Alignment is also often performed by inspection and manual alignment. In one class of embodiments, the polypeptides herein are at least 70%, generally at least 75%, optionally at least 80%, 85%, 90%, 95% or 99% or more identical to a reference polypeptide, as measured by BLASTP (or CLUSTAL, or any other available alignment software) using default parameters. Similarly, nucleic acids can also be described with reference to a starting nucleic acid, e.g., they can be 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more identical to a reference nucleic acid, as measured by BLASTN (or CLUSTAL, or any other available alignment software) using default parameters.

[0026] The terms “substantially identical” nucleic acid or amino acid sequences means that a nucleic acid or amino acid sequence comprises a sequence that has at least 90% sequence identity or more, preferably at least 95%, more preferably at least 98% and most preferably at least 99%, compared to a reference sequence using the programs described above (preferably BLAST) using standard parameters. For example, the BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)). Percentage of sequence identity is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues. In a most

preferred embodiment, the sequences are substantially identical over the entire length of the coding regions.

[0027] The term “nucleic acid” or “polynucleotide” refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in manner similar to naturally occurring nucleotides. A “polynucleotide sequence” is a nucleic acid (which is a polymer of nucleotides (A,C,T,U,G, etc. or naturally occurring or artificial nucleotide analogues) or a character string representing a nucleic acid, depending on context. Either the given nucleic acid or the complementary nucleic acid can be determined from any specified polynucleotide sequence.

[0028] The term “modulate” with respect to biological activities of a TRPV2 molecule refers to a change in the cellular level, subcellular localization or other biological activities of TRPV2 (e.g., its ion channel activity). Modulation of TRPV2 activities can be up-regulation (i.e., activation or stimulation) or down-regulation (i.e. inhibition or suppression). For example, modulation may cause a change in cellular level or subcellular localization of TRPV2, enzymatic modification (e.g., phosphorylation) of TRPV2, binding characteristics (e.g., binding to a substrate or ATP), or any other biological, functional, or immunological properties of TRPV2 proteins. The change in activity can arise from, for example, an increase or decrease in expression of the TRPV2 gene, the stability of mRNA that encodes the TRPV2 protein, translation efficiency, or from a change in other bioactivities of the TRPV2 enzymes (e.g., its ion channel activity). The mode of action of a TRPV2 modulator can be direct, e.g., through binding to the TRPV2 protein or to a gene encoding the TRPV2 protein. The change can also be indirect, e.g., through binding to and/or modifying (e.g., enzymatically) another molecule which otherwise modulates TRPV2.

[0029] The term “operably linked” refers to a functional relationship between two or more polynucleotide (e.g., DNA) segments. Typically, it refers to the functional relationship of a transcriptional regulatory sequence to a transcribed sequence. For example, a TRPV2 promoter or enhancer sequence, is operably linked to a coding sequence if it stimulates or modulates the transcription of the coding sequence in an appropriate host cell or other expression system. Generally, promoter transcriptional regulatory sequences

that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, i.e., they are cis-acting. However, some transcriptional regulatory sequences, such as enhancers, need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance. A polylinker provides a convenient location for inserting coding sequences so the genes are operably linked to the TRPV2 promoter. Polylinkers are polynucleotide sequences that comprise a series of three or more closely spaced restriction endonuclease recognition sequences.

[0030] The term "polypeptide" is used interchangeably herein with the terms "polypeptides" and "protein(s)", and refers to a polymer of amino acid residues, e.g., as typically found in proteins in nature. A "mature protein" is a protein which is full-length and which, optionally, includes glycosylation or other modifications typical for the protein in a given cell membrane.

[0031] Transcription refers to the process involving the interaction of an RNA polymerase with a gene, which directs the expression as RNA of the structural information present in the coding sequences of the gene. The process includes, but is not limited to the following steps: (1) transcription initiation, (2) transcript elongation, (3) transcript splicing, (4) transcript capping, (5) transcript termination, (6) transcript polyadenylation, (7) nuclear export of the transcript, (8) transcript editing, and (9) stabilizing the transcript.

[0032] A transcription regulatory element or sequence include, but is not limited to, a promoter sequence (e.g., the TATA box), an enhancer element, a signal sequence, or an array of transcription factor binding sites. It controls or regulates transcription of a gene operably linked to it.

[0033] A "variant" of a molecule such as a TRPV2 is meant to refer to a molecule substantially similar in structure and biological activity to either the entire molecule, or to a fragment thereof. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not identical to that found in the other, or if the sequence of amino acid residues is not identical.

III. Screening for TRPV2 Modulators: General Scheme

[0034] TRPV2 is also called vanilloid receptor-like protein 1 (VRL-1). It is a member of the TRPV family of proteins and is a homolog of the capsaicin/vanilloid receptor (VR1, or TRPV1). Although TRPV2 does not bind capsaicin, like TRPV1 it is activated by noxious heat ($>52^{\circ}\text{C}$). TRPV2 is expressed in a subset of medium- to large-diameter neurons, e.g., sensory neurons in the dorsal root ganglion. However, its expression is not limited to the sensory nervous system. Expression is also observed in tissues other than sensory ganglia and spinal cord, including lung, spleen, intestine, and brain (Caterina et al., *Nature* 398: 436 – 441, 1999).

[0035] In addition to being a noxious heat sensor, TRPV2 was also found to be a stretch sensor in vascular smooth muscles. It was shown TRPV2 plays a role in activation of a nonselective cation channel current (NSCC) and elevated intracellular Ca^{2+} level. Recombinant expression of TRPV2 in CHO cells showed that it can be activated by membrane stretch as well as hypotonic stimulation and is responsible for elevation of intracellular Ca^{2+} level by cell stretch (Muraki et al., *Circ Res.* 93: 829-38, 2003). It was also reported that TRPV2 is phosphorylated by the cAMP-dependent kinase (PKA) in vitro (Stokes et al., *J Exp Med.*, 200:137-47, 2004). Further, Barnhill et al. (*J Cell Biochem.* 91:808-20, 2004) described the interaction of TRPV2 channel with the RGA gene product.

[0036] As detailed in the following sections, in some methods of the present invention, novel modulators of T cell activation and function are identified by screening test compounds for ability to modulate (e.g., inhibit) TRPV2-mediated calcium influx or mobilization in a T cell. Because calcium influx is an essential step to T cell activation, compounds thus identified will be able to modulate T cell activities. As a control, the compounds can also be examined for ability to modulate a cell that does not express TRPV2 (e.g., a T cell which has TRPV2 knockout).

[0037] In some other methods, test compounds are first screened for ability to modulate a biological activity of a TRPV2. The biological activities of TRPV2 to be monitored in the screening assays can be any of the activities described above, e.g., its cation channel related activities or its phosphorylation. The biological activities of TRPV2 to be monitored can also be its expression or its cellular level, as well as a specific binding of TRPV2 to a test compound. After test compounds that modulate a biological activity of TRPV2 have been identified, they are typically further examined for ability to modulate a

biological activity related to T cell activation and function (e.g., T cell proliferation or differentiation). This step serves to confirm that by modulating the biological activity of TRPV2, compounds identified in the first step can indeed regulate (e.g., inhibit) T cell activation and function. These methods can also additionally include a control step to examine the compounds with cells that do not express TRPV2.

[0038] TRPV2 from various species can be employed in screening the TRPV2 modulators of the present invention. These include TRPV2 encoded by polynucleotides with accession numbers AJ487963, AF129112 and NM_016113 (human); NM_011706 (mouse); NM_017207 and AF129113 (rat); and AY487844 (rat/mouse fusion cell line). Preferably, a human TRPV2 molecule is used. Examples of TRPV2 polypeptide sequences include amino acid sequences with Accession Nos. NP_057197 and CAD32310 (human); Accession Nos. NP_035836 and AAH05415 (mouse); Accession No. NP_058903 (rat), and Accession No. AAS66752 (rat/mouse fusion cell line). Any of these TRPV2 sequences or substantially identical sequences thereof can be employed in the screening assay to identify TRPV2 modulators in the present invention.

[0039] In addition to an intact TRPV2 molecule or a polynucleotide encoding the intact TRPV2 molecule, a TRPV2 fragment, analog, or a functional derivative can also be used. The TRPV2 fragments that can be employed in these assays usually retain one or more of the biological activities of the TRPV2 molecule (typically, its ion channel activity or its phosphorylation by a cAMP dependent kinase). As noted above, TRPV2s from the different species have already been sequenced and well characterized. Therefore, their fragments, analogs, derivatives, or fusion proteins can be easily be obtained using methods well known in the art. For example, a functional derivative of a TRPV2 can be prepared from a naturally occurring or recombinantly expressed protein by proteolytic cleavage followed by conventional purification procedures known to those skilled in the art. Alternatively, the functional derivative can be produced by recombinant DNA technology by expressing only fragments of a TRPV2 that retain its ion channel activity.

IV. Test Compounds

[0040] Test agents that can be screened with methods of the present invention include polypeptides, beta-turn mimetics, polysaccharides, phospholipids, hormones,

prostaglandins, steroids, aromatic compounds, heterocyclic compounds, benzodiazepines, oligomeric N-substituted glycines, oligocarbamates, polypeptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Some test agents are synthetic molecules, and others natural molecules.

[0041] Test agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. Combinatorial libraries can be produced for many types of compound that can be synthesized in a step-by-step fashion. Large combinatorial libraries of compounds can be constructed by the encoded synthetic libraries (ESL) method described in WO 95/12608, WO 93/06121, WO 94/08051, WO 95/35503 and WO 95/30642. Peptide libraries can also be generated by phage display methods (see, e.g., Devlin, WO 91/18980). Libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts can be obtained from commercial sources or collected in the field. Known pharmacological agents can be subject to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

[0042] Combinatorial libraries of peptides or other compounds can be fully randomized, with no sequence preferences or constants at any position. Alternatively, the library can be biased, i.e., some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in some cases, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, or to purines.

[0043] The test agents can be naturally occurring proteins or their fragments. Such test agents can be obtained from a natural source, e.g., a cell or tissue lysate. Libraries of polypeptide agents can also be prepared, e.g., from a cDNA library commercially available or generated with routine methods. The test agents can also be peptides, e.g., peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides can be digests of naturally occurring proteins, random peptides, or "biased" random peptides. In some methods, the test agents are polypeptides or proteins.

[0044] The test agents can also be nucleic acids. Nucleic acid test agents can be naturally occurring nucleic acids, random nucleic acids, or “biased” random nucleic acids. For example, digests of prokaryotic or eukaryotic genomes can be similarly used as described above for proteins.

[0045] In some preferred methods, the test agents are small molecules (e.g., molecules with a molecular weight of not more than about 1,000). Preferably, high throughput assays are adapted and used to screen for such small molecules. In some methods, combinatorial libraries of small molecule test agents as described above can be readily employed to screen for small molecule modulators of TRPV2s. A number of assays are available for such screening, e.g., as described in Schultz (1998) *Bioorg Med Chem Lett* 8:2409-2414; Weller (1997) *Mol Divers.* 3:61-70; Fernandes (1998) *Curr Opin Chem Biol* 2:597-603; and Sittampalam (1997) *Curr Opin Chem Biol* 1:384-91.

[0046] Libraries of test agents to be screened with the claimed methods can also be generated based on structural studies of the TRPV2 polypeptides, their fragments or analogs. Such structural studies allow the identification of test agents that are more likely to bind to the TRPV2 polypeptides. The three-dimensional structure of a TRPV2 polypeptide can be studied in a number of ways, e.g., crystal structure and molecular modeling. Methods of studying protein structures using x-ray crystallography are well known in the literature. See *Physical Bio-chemistry*, Van Holde, K. E. (Prentice-Hall, New Jersey 1971), pp. 221-239, and *Physical Chemistry with Applications to the Life Sciences*, D. Eisenberg & D. C. Crothers (Benjamin Cummings, Menlo Park 1979). Computer modeling of a TRPV2 polypeptide structure provides another means for designing test agents for screening TRPV2 modulators. Methods of molecular modeling have been described in the literature, e.g., U.S. Patent No. 5,612,894 entitled “System and method for molecular modeling utilizing a sensitivity factor”, and U.S. Patent No. 5,583,973 entitled “Molecular modeling method and system”. In addition, protein structures can also be determined by neutron diffraction and nuclear magnetic resonance (NMR). See, e.g., *Physical Chemistry*, 4th Ed. Moore, W. J. (Prentice-Hall, New Jersey 1972), and *NMR of Proteins and Nucleic Acids*, K. Wuthrich (Wiley-Interscience, New York 1986).

[0047] Modulators of the present invention also include antibodies that specifically bind to a TRPV2 polypeptide. Such antibodies can be monoclonal or polyclonal. Such

antibodies can be generated using methods well known in the art. For example, the production of non-human monoclonal antibodies, e.g., murine or rat, can be accomplished by, for example, immunizing the animal with a TRPV2 polypeptide or its fragment (See Harlow & Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor New York). Such an immunogen can be obtained from a natural source, by peptides synthesis or by recombinant expression.

[0048] Humanized forms of mouse antibodies can be generated by linking the CDR regions of non-human antibodies to human constant regions by recombinant DNA techniques. See Queen et al., *Proc. Natl. Acad. Sci. USA* 86, 10029-10033 (1989) and WO 90/07861. Human antibodies can be obtained using phage-display methods. See, e.g., Dower et al., WO 91/17271; McCafferty et al., WO 92/01047. In these methods, libraries of phage are produced in which members display different antibodies on their outer surfaces. Antibodies are usually displayed as Fv or Fab fragments. Phage displaying antibodies with a desired specificity are selected by affinity enrichment to a TRPV2 polypeptide of the present invention.

[0049] Human antibodies against a TRPV2 polypeptide can also be produced from non-human transgenic mammals having transgenes encoding at least a segment of the human immunoglobulin locus and an inactivated endogenous immunoglobulin locus. See, e.g., Lonberg et al., WO93/12227 (1993); Kucherlapati, WO 91/10741 (1991). Human antibodies can be selected by competitive binding experiments, or otherwise, to have the same epitope specificity as a particular mouse antibody. Such antibodies are particularly likely to share the useful functional properties of the mouse antibodies. Human polyclonal antibodies can also be provided in the form of serum from humans immunized with an immunogenic agent. Optionally, such polyclonal antibodies can be concentrated by affinity purification using a TRPV2 polypeptide or its fragment.

V. Screen Test Compounds for TRPV2 Modulators

To identify modulators of T cell activation and function, test compounds are first screened for ability to modulate a biological activity of TRPV2 as described herein. In some preferred embodiments, test compounds are examined for ability to modulate (e.g., inhibit) a cation channel activity or whole cell current in cells expressing TRPV2 (e.g.,

elevating intracellular calcium level). Many cell types expressing TRPV2 can be employed in this screening step. They can be cells that express TRPV2 endogenously, e.g., a T cell (e.g., CD4⁺ or CD8⁺ T cells) or a mast cell. They can also be cells that recombinantly express TRPV2. As detailed below, many mammalian cells may be used to recombinantly express TRPV2, e.g., CHO cell, HEK cell, or *Xenopus* oocyte into which a polynucleotide encoding TRPV2 has been introduced or transiently transfected. If a T cell is employed to examine test compounds for modulators of TRPV2 mediated calcium influx in this screening step, the additional screening step outlined above may or may not be needed.

[0050] Various assays are known in the art which can be employed in the present invention to measure whole cell current or calcium influx in a cell. For example, TRPV2-dependent mobilization of Ca²⁺ in a T cell line (e.g., Jurkat cells) can be monitored as described in the Examples below. Effect of test compounds on low-dose Ionomycin, Thapsigargin or 2-Aminoethoxydiphenyl Borate (2APB)-activated whole cell currents in HEK293 or CHO cells transiently expressing human or mouse TRPV2 can be examined as described in Hu et al., *J. Biol. Chem.*, 279: 35741-35748, 2004 and in other publications. Changes of intracellular calcium levels can also be observed directly at the single cell level using ratiometric measurement of the fluorescence intensities of fluorescent dyes as reported in Donnadieu et al., *Curr. Biol.* 4: 584-595, 1994. Other assays for monitoring calcium flux or intracellular calcium concentration include the method using a photomultiplier-based system to monitor fluorescence that was described in Turner et al. (*Biochem. J.* 371: 341-350, 2003); and the microscopy or flow cytometry based method that was described in Chen et al. (*J Exp Med.* 197:1689-99, 2003). Calcium mobilization can also be monitored via patch-clamping and electrophysiological recording of single channel currents in T cells as described for TRPM4 in Launay et al., *Science* 306: 1374-1377, 2004 and also elsewhere in the literature for other channels, including CRAC channels.

[0051] In some embodiments, cDNAs expressing TRPV2 can be introduced into *Xenopus* oocytes (e.g. via microinjection), followed by electrophysiological monitoring of the effects of TRPV2 expression on calcium currents in *Xenopus* oocytes.

[0052] Test compounds can also be monitored for any effect on activation of TRPV2 channel currents by membrane stretch and hypotonic stimulation in TRPV2-CHO cells (see, e.g., Muraki et al., *Cir. Res.*, 93:829, 2003). This method is based on the observation that

TRPV2 is sensitive to membrane stretch induced by negative pressure through patch pipettes, and this involves a marked elevation of Ca^{2+} level. TRPV2 activity is monitored by recording single channel current of TRPV2 activated by hypotonic stimulation. Any of these assays can be readily adopted in the present invention to screen for modulators of the cation channel activity of TRPV2.

[0053] In some other embodiments, test compounds can be screened for their ability to modulate other biochemical activities of TRPV2. As noted above, TRPV2 is phosphorylated by a cAMP-dependent kinase (PKA). Therefore, test compounds may be screened for ability to modulate TRPV2 phosphorylation by PKA in vitro. This can be performed as described in Stokes et al. (J Exp Med., 200:137-47, 2004).

[0054] In some embodiments, test compounds can be first screened for their ability to bind to a TRPV2 polypeptide. Compounds thus identified can be further subject to assay for ability to modulate (e.g., to inhibit) TRPV2 ion channel activity as described above. Binding of test agents to a TRPV2 polypeptide can be assayed by a number of methods including, e.g., labeled in vitro protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, functional assays (phosphorylation assays, etc.), and the like. See, e.g., U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168; and also Bevan et al., Trends in Biotechnology 13:115-122, 1995; Ecker et al., Bio/Technology 13:351-360, 1995; and Hodgson, Bio/Technology 10:973-980, 1992. The test agent can be identified by detecting a direct binding to the TRPV2 polypeptide, e.g., co-immunoprecipitation with the TRPV2 polypeptide by an antibody directed to the TRPV2 polypeptide. The test agent can also be identified by detecting a signal that indicates that the agent binds to the TRPV2 polypeptide, e.g., fluorescence quenching or fluorescence polarization.

[0055] In some other methods, test agents are assayed for activity to modulate expression or cellular level of TRPV2, e.g., transcription, translation, or post-translational modification. Various biochemical and molecular biology techniques well known in the art can be employed to practice the present invention. Such techniques are described in, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, N.Y., Second (1989) and Third (2000) Editions; and Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York (1987-1999). In some

embodiments, endogenous levels of a TRPV2 can be directly monitored in cells normally expressing TRPV2 (e.g., T cells). In some embodiments, expression or cellular level of a TRPV2 can be examined in an expression system using cloned cDNA or genomic sequence encoding the TRPV2.

[0056] Alternatively, modulation of expression of a TRPV2 gene can be examined in a cell-based system by transient or stable transfection of an expression vector into cultured cell lines. Assay vectors bearing transcription regulatory sequences (e.g., promoter) of a TRPV2 gene operably linked to reporter genes can be transfected into any mammalian host cell line for assays of promoter activity. Constructs containing a TRPV2 gene (or a transcription regulatory element of a TRPV2 gene) operably linked to a reporter gene can be prepared using only routinely practiced techniques and methods of molecular biology (see, e.g., Sambrook et al. and Ausubel et al., *supra*). General methods of cell culture, transfection, and reporter gene assay have been described in the art, e.g., Ausubel, *supra*; and Transfection Guide, Promega Corporation, Madison, WI (1998). Any readily transfectable mammalian cell line may be used to assay TRPV2 promoter function or to express TRPV2, e.g., CHO, COS, HCT116, HEK 293, MCF-7, and HepG2 are all suitable cell lines.

[0057] When inserted into the appropriate host cell, the transcription regulatory elements in the expression vector induces transcription of the reporter gene by host RNA polymerases. Reporter genes typically encode polypeptides with an easily assayed enzymatic activity that is naturally absent from the host cell. Typical reporter polypeptides for eukaryotic promoters include, e.g., chloramphenicol acetyltransferase (CAT), firefly or Renilla luciferase, beta-galactosidase, beta-glucuronidase, alkaline phosphatase, and green fluorescent protein (GFP).

VI. Screen for Compounds that Modulate T Cell Activation and Function

[0058] The present invention provides compositions and methods for modulating T cell activation and functions. As a consequence of the connection between TRPV2 and T cell activation, modulation of biological activities or cellular levels of TRPV2 can lead to modulation of T cell function as well as immune responses mediated by T cells. To identify novel modulators of T cells, a TRPV2 modulator described above can be further examined

to confirm its ability to modulate T cell activation and function. This typically involves testing the compounds for ability to modulate an activity related to T cell activation and function ("T cell activation related activity").

[0059] In some embodiments, the T cell activation related activity tested in this screening step is associated with T cell proliferation. T cell proliferation generally refers to the clonal expansion of a T cell in response to various stimuli, e.g., as a result of antigen-specific T cell activation. Many methods and assays are available in the art which can be employed and modified to assess modulating activities of test compounds on T cell proliferation. T cell proliferation can be monitored by examining cell size or by assaying for expression of cell surface molecules in response to exposure to a ligand or other agents, as described in US Patent No. 6,534,055. Another commonly practiced in vitro method for measuring T cell proliferation is the ^3H -thymidine incorporation assay described in Bloemena et al., J. Immunol. Methods 122:161-167, 1989; and Chen et al., J Exp Med. 197:1689-99, 2003. T cell proliferation can be determined with this method based on incorporation of ^3H -thymidine into newly generated DNA. In addition, Messele et al. (Clin Diagn Lab Immunol. 7:687-92, 2000) described techniques of assaying in vitro proliferative activity of CD3+ peripheral blood mononuclear cells in response to appropriate stimuli. In some methods, T cell activation is measured by flow cytometric assessment of CD38 expression on T cells. In some methods, T cell proliferative activity can be determined by an enzyme-linked immunosorbent assay (ELISA) based on bromo-2'-deoxyuridine (BrdU) incorporation. In some other methods, proliferation of CD4+ T cells can be monitored using the Cell Titer 96™ non-radioactive cell proliferation system manufactured by Promega.

[0060] In some other embodiments, the T cell activation related activity examined is an activity associated with T cell differentiation. T cell differentiation associated activities can include any of the biological activities associated with T cell development at the various stages of the process in which the CD4⁻CD8⁻ double negative progenitor cells develop into mature CD4⁺ or CD8⁺ T cells. Methods for studying T cell differentiation are well known in the art. For example, differentiation of T cells at different stages can be examined with cell suspensions isolated from mouse thymus, spleen, peripheral blood or lymph nodes. T cells of different developmental stages can be readily quantified using antibodies against

specific surface markers by, e.g., flow cytometry and BrdU labeling (see, e.g., Rodriguez-Borlado et al., *J Immunol.* 170:4475-82, 2003; and Kufel et al., *Cancer Immunol.* 1:10, 2001).

[0061] In some methods, T cell differentiation activities to be monitored in the screening methods are activities relating to the developmental stage during which the CD4⁺CD8⁺ double positive T cells develop into CD4⁺ or CD8⁺ single positive T cells. They can also be the further differentiation of CD4⁺ T cells into Th1, Th2, or natural occurring or induced T_{reg} cells (CD4⁺CD25⁺ T cells, T_{reg}, Th3, Tr1, T_s cells), or the development of CD8⁺ cells into subtypes of differing physiological activities (T_{CT}1, T_{CT}2 etc.). Th1 and Th2 helper T cells have distinct patterns of cytokine expression. Th1 cells produce interleukin (IL)-2, interferon (IFN)- γ , tumor necrosis factor (TNF)- α , lymphotoxin, and etc. Th2 cells secrete IL-4, IL-5, IL-10, IL-13, and etc., and are particularly responsible for governing allergy and humoral immunity. T_{reg} cells negatively regulate the function of other T cells via secreting IL-10 and other activities. Inappropriate activity or localization of T_{reg} cells has been linked to cancer, whereas impairment of T_{reg} function has been linked to autoimmunity, e.g., diabetes or rheumatoid arthritis (Sutmuller et al., *Drug Discov. Today.* 9: 310-6, 2004; Fehervari and Sakaguchi, *Curr. Op. Immunol.* 16: 203-208, 2004; Sakaguchi, *Ann. Rev. Immunol.* 22: 531-562, 2004; and Terabe and Berzofsky, *Curr. Op. Immunol.* 16: 157-162, 2004).

[0062] Methods for assaying differentiation activities of various T cell types are known in the art. For example, T cell differentiation towards Th2 lineage can be monitored by measuring Th2 cytokine production (e.g., IL-4, IL-5 and IL-13) as described in Chen et al., *J Exp Med.* 197: 1689-99, 2003; Banning et al., *Methods Mol Biol.* 215:15-22, 2003; and Fiorentino et al., *J. Exp. Med.* 170: 2081-2095, 1989. Similarly, Th1 cell differentiation can also be analyzed using the cytokine assays and/or antibody staining for IFN γ or TNF α , e.g., as described in Patel et al., *J Immunol.* 173: 5501-8, 2004; Bajenoff et al., *J Immunol.* 171: 6355-62, 2003; and Bird et al., *Immunity* 9:229-237, 1998. Differentiation into T_{reg} cells can be examined via monitoring expression of Foxp3 within T_{reg} cells, expression of the surface markers CD4, CD25 or GITR, or via monitoring of the production of IL-10, TGF β or other cytokines (see, e.g., Sutmuller et al., *Drug Discov Today* 9: 310-6, 2004; Fehervari and Sakaguchi, *Curr. Op. Immunol.* 16: 203-208, 2004;

Sakaguchi, *Ann. Rev. Immunol.* 22: 531-562, 2004; and Terabe and Berzofsky, *Curr. Op. Immunol.* 16: 157-162, 2004).

[0063] In some embodiments, the T cell activation related activity assayed in the screening method is TRPV2 mediated calcium influx or elevation of intracellular calcium levels in a T cell. As noted above, the latter approach can be employed to directly identify compounds that modulate T cell activation and function without first screening test compounds for modulation of other biological activities of TRPV2. TRPV2 mediated calcium mobilization or influx in a T cell can be measured using the methods described above. Additional assays for monitoring intracellular calcium concentration during T cell activation include the calcium imaging method using a calcium-sensitive fluorescent dye as described in Wülfing et al., *J. Exp. Med.* 185: 1815-1825, 1997.

VII. Therapeutic Applications

[0064] The ability to specifically regulate T cell activation and function provides particularly useful therapeutic applications of the present invention. For example, via specific and selective inhibition of a TRPV2 molecule (e.g., TRPV2) which results in inhibition of T cell maturation, activation and function, the T cell-modulating compounds of the present invention can lead to profound immunosuppression. TRPV2 specifically accumulates only in the brain and in T cells or lymphoid tissues containing high numbers of T cells. Therefore, selective TRPV2 inhibitors could prove highly tissue and even cell type specific. This will reduce the likelihood of adverse side reactions and general toxicity. As a result, therapeutic compositions comprising selective TRPV2-inhibitors of the present invention are advantageous over currently used immunosuppression drugs such as cyclosporine. The latter has severe side effects due to its pleiotropic action.

[0065] A number of diseases and conditions due to abnormal T cell development or functions can be prevented or treated with methods and compositions of the present invention. For example, many diseases can potentially be ameliorated by immunosuppression (e.g., inhibiting T cell activation). Examples of such diseases include, but are not limited to, inflammation, autoimmune diseases (e.g., systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis), psoriasis, graft-versus-host disease, or allergic diseases (e.g., asthma, rhinitis, COPD, and dermatitis). Subjects with

tissue or organ transplants can be treated with compounds that inhibit T cell activation in order to prevent graft rejection. Conversely, compounds that stimulate T cell activation and function are useful to ameliorate certain diseases, or conditions, such as immunodeficiency diseases or cancer. Also, compounds that specifically inhibit T_{reg} cell function in tumors could provide treatments for cancer. In addition to treating these diseases or conditions, TRPV2 modulators of the present invention (e.g., TRPV2 inhibitors) are also useful for preventing or modulating the development of such diseases or disorders in a subject (including human and animals such as other mammals) suspected of being, or known to be, prone to such diseases or disorders.

[0066] The TRPV2 modulators of the present invention can be directly administered under sterile conditions to the subject to be treated. The modulators can be administered alone or as the active ingredient of a pharmaceutical composition. The therapeutic composition of the present invention can also be combined with or used in association with other therapeutic agents. In some applications, a TRPV2-modulating compound of the present invention may be used in conjunction with known immunosuppressive drugs such as cyclosporine, FK506 or glucocorticoids, or in combination with cytostatic drugs.

[0067] Pharmaceutical compositions of the present invention typically comprise at least one active ingredient together with one or more acceptable carriers thereof. Pharmaceutically carriers enhance or stabilize the composition, or to facilitate preparation of the composition. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered (e.g., nucleic acid, protein, modulatory compounds or transduced cell), as well as by the particular method used to administer the composition. They should also be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the subject. This carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral, sublingual, rectal, nasal, or parenteral. For example, the TRPV2-modulating compound can be complexed with carrier proteins such as ovalbumin or serum albumin prior to their administration in order to enhance stability or pharmacological properties.

[0068] There are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (see, e.g., *Remington: The Science and Practice of*

Pharmacy, Mack Publishing Co., 20th ed., 2000). Without limitation, they include syrup, water, isotonic saline solution, 5% dextrose in water or buffered sodium or ammonium acetate solution, oils, glycerin, alcohols, flavoring agents, preservatives, coloring agents, starches, sugars, diluents, granulating agents, lubricants, and binders, among others. The carrier may also include a sustained release material such as glyceryl monostearate or glyceryl distearate, alone or with a wax.

[0069] The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100% by weight. Therapeutic formulations are prepared by any methods well known in the art of pharmacy. The therapeutic formulations can be delivered by any effective means which could be used for treatment. See, e.g., Gilman et al., eds., Goodman and Gilman's: *The Pharmacological Bases of Therapeutics*, 8th ed., Pergamon Press, 1990; Remington: *The Science and Practice of Pharmacy*, Mack Publishing Co., 20th ed., 2000; Avis et al., eds., *Pharmaceutical Dosage Forms: Parenteral Medications*, published by Marcel Dekker, Inc., N.Y., 1993; Lieberman et al., eds., *Pharmaceutical Dosage Forms: Tablets*, published by Marcel Dekker, Inc., N.Y., 1990; and Lieberman et al., eds., *Pharmaceutical Dosage Forms: Disperse Systems*, published by Marcel Dekker, Inc., N.Y., 1990.

[0070] The therapeutic formulations can conveniently be presented in unit dosage form and administered in a suitable therapeutic dose. A suitable therapeutic dose can be determined by any of the well known methods such as clinical studies on mammalian species to determine maximum tolerable dose and on normal human subjects to determine safe dosage. Except under certain circumstances when higher dosages may be required, the preferred dosage of a TRPV2 modulator usually lies within the range of from about 0.001 to about 1000 mg, more usually from about 0.01 to about 500 mg per day.

[0071] The preferred dosage and mode of administration of a TRPV2 modulator can vary for different subjects, depending upon factors that can be individually reviewed by the treating physician, such as the condition or conditions to be treated, the choice of composition to be administered, including the particular TRPV2 modulator, the age, weight, and response of the individual subject, the severity of the subject's symptoms, and the chosen route of administration. As a general rule, the quantity of a TRPV2 modulator

administered is the smallest dosage which effectively and reliably prevents or minimizes the conditions of the subjects. Therefore, the above dosage ranges are intended to provide general guidance and support for the teachings herein, but are not intended to limit the scope of the invention.

[0072] In some applications, a first TRPV2 modulator is used in combination with a second TRPV2 modulator in order to modulate T cell development and function to a more extensive degree than cannot be achieved when one TRPV2 modulator is used individually.

EXAMPLES

[0073] The following examples are offered to illustrate, but not to limit the present invention.

Example 1 Expression of TRPV2 in T cells and other cells of the immune system

[0074] We surveyed the GNF gene expression database (symatlas.gnf.org) for candidate ion channels which show an accumulation of their mRNA levels in T or B cells and tissues of the immune system. We found that the mRNA of the TRP ion channel family member TRPV2 accumulates strongly in sorted CD4+ or CD8+ peripheral T cells, peripheral B cells, as well as in tissues of the immune system in humans and mice. In humans, we found additional accumulation of TRPV2 in Natural Killer cells, myeloid cells, monocytes and dendritic cells (for details, see symatlas.gnf.org). Analyses of TRPV2 expression via TaqMan RT-PCR confirmed its presence in Jurkat cells and primary human T cells (Fig. 1).

[0075] In order to investigate potential functions for TRPV2 in T cells, we employed RNA-interference (RNAi) approaches to disrupt expression of the TRPV2 mRNA in Jurkat cells. Nucleofection of expression constructs for either one of four different small hairpin DNAs (shDNAs) targeting different regions of the human TRPV2 mRNA resulted in variable degrees of reduction of TRPV2 mRNA expression in Jurkat cells (Fig. 1). In contrast, none of the four constructs affected expression of GAPDH significantly, indicating that our hTRPV2 shDNAs do not suppress mRNA expression in a non-specific, global

manner. hTRPV2.1 and hTRPV2.4 reproducibly provided the highest knockdown efficiency, typically resulting in a more than 60% reduction of TRPV2 mRNA levels. They were therefore used for all further studies, together with appropriate controls.

Example 2. TRPV2 mediates calcium influx and signaling in T cells

[0076] In neurons, TRPV2 is activated by noxious heat above 52°C. In order to gain evidence for a functionally relevant expression of TRPV2 in T cells, we therefore tested whether temperature elevation can elicit Ca^{2+} mobilization in Jurkat cells. We found that, indeed, re-addition of extracellular Ca^{2+} at temperatures above 25°C resulted in a significant Ca^{2+} influx in eGFP negative, positive or high cells containing the pGL3 control shDNA construct. The influx peaked around 48°C, close to the threshold temperature for TRPV2 gating in neurons. At higher temperatures, significant influx still occurs. In cells harboring the TRPV2.1 shDNA, only the eGFP negative fraction displayed a Ca^{2+} influx profile very similar to that of the pGL3 containing control cells. In contrast, eGFP positive or high cells, in which the hTRPV2 mRNA should be knocked down, showed strongly impaired heat induced Ca^{2+} mobilization. These data show that heat can induce Ca^{2+} mobilization in Jurkat T cells, demonstrating that these cells express temperature sensitive Ca^{2+} channels. Moreover, the fact that knockdown of TRPV2 essentially abrogated this effect reveals that TRPV2 is expressed in Jurkat cells and could play an important role in mediating Ca^{2+} signaling in T cells.

[0077] We next conducted a series of experiments in which the effect of hTRPV2-knockdown on TCR mediated Ca^{2+} mobilization in T cells was investigated. Jurkat cells were nucleofected with expression constructs harboring validated shDNAs against hTRPV2 (hTRPV2.1, hTRPV2.4), or the controls Lck (Lck) or firefly luciferase (pGL3) along with eGFP expression constructs. 3 days post nucleofection, cells were labeled with Indo-1 and then stimulated with anti-TCR antibodies (OKT3) or Thapsigargin (Tg, a compound that triggers Ca^{2+} release from intracellular stores downstream of the TCR via inhibition of SERCA) or medium (baselines) in the absence of extracellular Ca^{2+} (and, in some cases, also Mg^{2+}). The intracellular Ca^{2+} concentration was monitored over time in cells expressing no eGFP (enriched for un-nucleofected cells which should show no shDNA mediated target gene knockdown), low levels of eGFP (enriched for low-level nucleofected

cells expected to show low-level target gene knockdown) or high levels of eGFP (enriched for high-level nucleofected cells expected to show the highest degree of target gene knockdown). In the absence of extracellular Ca^{2+} , OKT3 or Tg treatment results in a transient elevation of the cytoplasmic Ca^{2+} concentration via store release, but no sustained Ca^{2+} influx can occur.

[0078] As expected after knockdown of an essential mediator of TCR signaling, eGFP high cells harboring the Lck shDNA showed a reduction in OKT3-induced store release compared to eGFP high cells harboring the pGL3 control vector, or to eGFP negative cells. Interestingly, eGFP high cells harboring the hTRPV2.1 shDNA displayed a comparable reduction in store release, which could be indicative of impaired conformational coupling or other functional interactions between CRAC channels and IP3 receptors.

[0079] After return of Ca^{2+} levels to near baseline values, Ca^{2+} (and in some cases also Mg^{2+}) was re-added to the extracellular buffer, which resulted in an immediate capacitative Ca^{2+} influx in control cells. This Ca^{2+} influx was drastically reduced in OKT3-stimulated eGFP high cells harboring shDNAs against Lck or hTRPV2, but not in eGFP negative cells, compared to pGL3 nucleofected cells. Due to differing nucleofection or shDNA expression levels or RNAi efficiency, the amount of reduction differed between experiments and between hTRPV2.1 and hTRPV2.4. In some experiments, it was similar to the degree of reduction obtained after Lck knockdown for either or both hTRPV2 shDNAs. Moreover, some experiments revealed a reduction in Ca^{2+} influx by more than 60%. These data show that shDNA mediated knockdown of TRPV2 results in a significant impairment of TCR-induced Ca^{2+} influx via the plasma membrane, and that this defect may result in a concomitant defect in store release, presumably via impaired conformational coupling.

[0080] When cells were stimulated with Thapsigargin (Tg) instead of OKT3, the Lck shDNA had no effect, as expected. However, eGFP high cells harboring shDNAs against hTRPV2 showed significant impairment of the sustained Ca^{2+} influx. Some heterogeneity was again observed between different experiments and between both hTRPV2 shDNAs, likely due to differing nucleofection or shDNA expression levels or RNAi efficiency. This notwithstanding, the degree of impairment was usually higher in Tg stimulated cells than in OKT3-stimulated cells, frequently reaching up to 80% reduction in Ca^{2+} influx upon Ca^{2+}

re-addition. These data show that knockdown of hTRPV2 specifically impairs the Ca^{2+} release activated Ca^{2+} influx in Jurkat T cells.

[0081] In other experiments, we kept the cells in medium with or without extracellular Ca^{2+} and stimulated with OKT3 or Tg. Complementing the results from our Ca^{2+} re-addition experiments, we found impaired OKT-3 induced Ca^{2+} mobilization upon knockdown of either Lck or hTRPV2, and impaired Tg-induced Ca^{2+} mobilization only upon knockdown of hTRPV2. In all experiments, the final addition of Ionomycin in the presence of extracellular Ca^{2+} resulted in a massive Ca^{2+} mobilization in all cells, indicating that the cells were intact and showed similar capacities to non-specifically mobilize Ca^{2+} .

Example 3. Inhibition of calcium influx by dominant negative TRPV2 mutants

[0082] We further examined the effect of dominant TRPV2 negative mutants on calcium influx in response to T cell receptor signaling. It was known that replacement of a negative charge in the pore domain of TRPV channels (usually through glutamic acid to alanine mutation) abrogates Ca^{2+} conductance and can generate dominant negative (dn) TRPV channels (Cui et al., J Biol Chem 277: 47175-47183, 2002). Expression of these dnTRPVs can inhibit agonist induced Ca^{2+} mobilization.

[0083] In order to generate a dominant negative human TRPV2 channel (TRIA-hTRPV2), we replaced the amino acid triplet L₆₁₁E₆₁₂L₆₁₃ with an AAA-triplet (TRIA). The TRIA-hTRPV2 mutant was generated via PCR mutagenesis. PCR product containing the mutation was used to replace the corresponding Kpn1/Xho1 fragment in pCRTOP02.1hTRPV2, which contained wildtype hTRPV2. This generated pCRTOP0-TRIAhTRPV2, which was subsequently subcloned via Spe1/Xba1 digest into pEF1A, generating pEF1ahTRPV2PM. The STOP codon was then removed via PCR amplification, followed by TOPO-subcloning of the PCR product and final insertion into pEF1-MycHisA, generating pEF1-MycHisA-TRIAhTRPV2 #4-11. In parallel, the same PCR strategy was used to generate wildtype hTRPV2 encoding pEF1-MycHisA-wthTRPV2 #10-13. DNA sequence analysis unveiled two non-conservative artificial mutations in pEF1-MycHisA-TRIAhTRPV2 #4-11 which were subsequently removed by replacing the pEF1-MycHisA-TRIAhTRPV2 #4-11 Not1/Ale1 862 bp fragment with the corresponding fragment of pEF1-MycHisA-wthTRPV2 #10-13, which was found to be identical with the wt sequence. This

finally resulted in the generation of pEF1-HisMyc-TRIAhTRPV2 #1, which harbors a TRIA-mutant that is otherwise identical to wildtype pEF1-MycHisA-wthTRPV2 #10-13. Sequences of the wildtype TRPV2 and the mutant TRPV2 molecules were confirmed by sequence analysis.

[0084] We then generated and analyzed stably transfected Jurkat cell clones expressing the dominant TRIA-hTRPV2 mutant. Jurkat T cells were transfected with pEF1-HisMyc-TRIAhTRPV2 #1 via electroporation using standard protocols. Subsequently, single cell derived clones were isolated via standard limiting dilution procedures. Clones 4H8, 2C12, 8A6, 2G4, 1F7 and 1B8 were tested for HisMyc-TRIAhTRPV2 expression via lysis and immunoblot analysis with an anti-Myc tag antibody. The 2C12, 1F7 and 1B8 clones were found to express high levels of HisMyc-TRIAhTRPV2, while the other clones had low levels of expression.

[0085] Subsequently, the effect of HisMyc-TRIAhTRPV2 expression on the ability of the cells to mobilize Ca^{2+} in response to T cell receptor stimulation was assessed using the OKT3-stimulation/ Ca^{2+} re-addition protocol previously described in the provisional application, using shDNA-mediated knockdown of Lck (Fig. 2., Lck-shDNA) as a positive control, and untransfected cells (J154 (wt)) as a negative control. We found that expression of HisMyc-TRIAhTRPV2 can affect OKT3-mediated Ca^{2+} mobilization to variable degrees. As demonstrated in the example shown in Fig. 2, strong expression in clones 1B8 (V2-TRIA1B8) or 1F7 (V2-TRIA1F7) correlates with intermediate to complete inhibition of OKT3-induced Ca^{2+} mobilization.

[0086] While the effect of HisMyc-TRIAhTRPV2 on TCR-induced Ca^{2+} mobilization is variable, our observation of significant to strong inhibition in several independently derived Jurkat T cell clones suggests that dominant negative TRIA-hTRPV2 can inhibit endogenous channels mediating Ca^{2+} influx and thereby T cell activation. Pharmacological inhibitors of TRPV2 can convert the endogenous, wildtype protein into a dominant negative by binding within the pore domain region, functionally mimicking the E>A substitution in the TRIA mutant. Therefore, pharmacological inhibition of TRPV2 can be pursued to inhibit T cell activation.

[0087] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

[0088] All publications, GenBank sequences, patents and patent applications cited herein are hereby expressly incorporated by reference in their entirety and for all purposes as if each is individually so denoted.

WE CLAIM:

1. A method for identifying an agent that modulates T lymphocyte activation and function, the method comprising assaying a biological activity of a transient receptor potential cation channel, subfamily V, member 2 (TRPV2) or a fragment thereof, in the presence of test compounds to identify a modulating agent that modulates the biological activity of the TRPV2.
2. The method of claim 1, further comprising testing the identified modulating agent for ability to modulate a T cell activation-related activity.
3. The method of claim 1, wherein the TRPV2 is human or murine TRPV2.
4. The method of claim 1, wherein the modulating agent inhibits the biological activity of TRPV2.
5. The method of claim 1, wherein the biological activity of TRPV2 is its phosphorylation or induction of whole cell current.
6. The method of claim 5, wherein TRPV2 phosphorylation activity is its phosphorylation by cAMP-dependent kinase (PKA).
7. The method of claim 5, wherein induction of whole cell current by TRPV2 is monitored by measuring intracellular calcium level in a cell transiently expressing TRPV2.
8. The method of claim 7, wherein the cell is a HEK293 cell or a CHO cell transiently transfected with a cDNA encoding human or murine TRPV2.
9. The method of claim 2, wherein the T cell activation-related activity is calcium influx into a cultured T cell.
10. The method of claim 9, wherein the T cell is a Jurkat cell, and calcium influx into the T cell is monitored by measuring intracellular calcium levels in the cell.

11. The method of claim 2, wherein the T cell activation-related activity is proliferation or differentiation of a T cell.
12. The method of claim 11, wherein the T cell is selected from the group consisting of thymus cells, CD4⁺CD8⁺ T cells, CD4⁺ T cells, CD8⁺ T cells, Th1, Th2, Treg, CD4⁺CD25⁺ T cells, T_{CT}1, T_{CT}2, Tr1, Ts, Tγδ, NKT cells.
13. The method of claim 2, wherein the T cell activation-related activity is inhibited by the modulating agent.
14. A method for identifying agents that modulate T cell activation and function, comprising assaying test compounds for ability to modulate calcium influx into a cell expressing transient receptor potential cation channel, subfamily V, member 2 (TRPV2), thereby identifying the agents that modulate T cell activation and function.
15. The method of claim 14, wherein the test compounds are assayed for ability to inhibit calcium influx into the cell.
16. The method of claim 14, wherein the cell is a T lymphocyte.
17. The method of claim 16, wherein the T lymphocyte is a cultured Jurkat cell.
18. The method of claim 14, wherein the cell is a cell transiently expressing TRPV2.
19. The method of claim 18, wherein the cell is a HEK293 or a CHO cell.
20. The method of claim 18, wherein the cell is transfected with a cDNA encoding human or murine TRPV2.
21. The method of claim 14, wherein calcium influx into the cell is monitored by measuring intracellular calcium level in the cell.

Figure 1

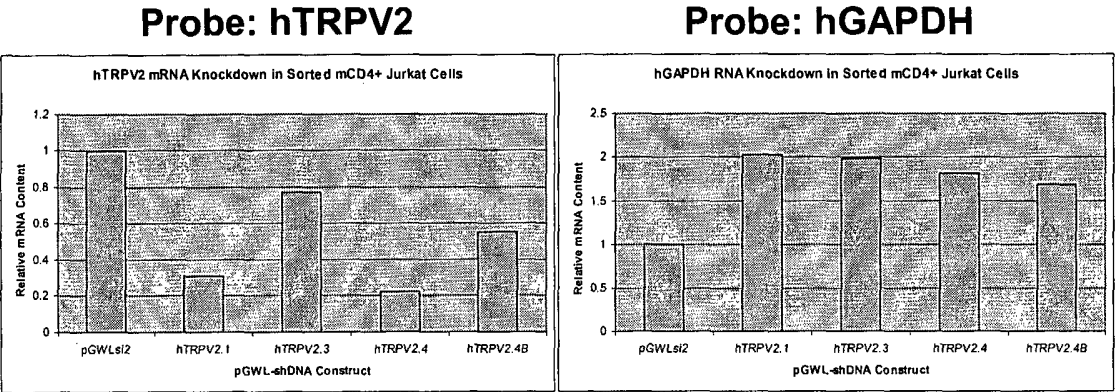


Figure 2

Ratio: Indo 1 (Violet)-A/Indo 1 (Blue)-A

