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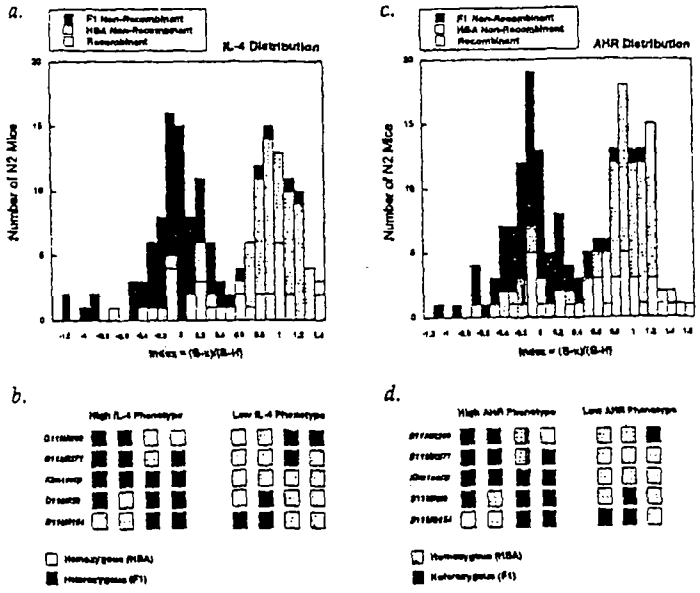

(57) Abstract: A genetic locus and corresponding family of proteins associated with regulation of immune function and cell survival are provided. These genes encode cell surface molecules with conserved IgV and mucin domains. The locus comprising the TIM family is genetically associated with immune dysfunction, including asthma. Furthermore, the TIM gene family is located within a region of human chromosome 5 that is commonly deleted in malignancies and myelodysplastic syndrome. Polymorphisms in the gene sequences are associated with the development of airway hyperreactivity and allergic inflammation, and T cell production of IL-4 and IL-13. The proteins include the human hepatitis A cellular receptor, *h*HAV*cr*-1.

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T CELL REGULATORY GENES AND METHODS OF USE THEREOF

BACKGROUND OF THE INVENTION

The astonishing complexity of the immune system is its greatest strength. The 10^{12}-10^{14} possible antibody specificities, the delicate interplay between the various regulatory and effector cells, the restriction of T cell responses according to MHC antigens; all these contribute to the ability of the host to effectively react against infectious agents and other antigens perceived as foreign. But this diversity has its drawbacks. Mistakes happen: the target of a response may turn out to be a normal self protein; inflammatory responses are misregulated; and normal responses are undesirably directed against grafts and transplanted cells. Under these circumstances, the complexity of the system makes diagnosis and therapy extremely difficult.

The profile of cytokines produced by $CD4^+$ T cells during an immune response determines the nature of effector functions which develop and regulates the outcome of an immune response. Production of IL-2 and IFN-γ during Th1-dominated responses is associated with vigorous cell-mediated immunity, the induction of IgG2a and inhibition of IgE synthesis, and with resistance to intracellular pathogens. In contrast, the production of IL-4, IL-5 and IL-10 during Th2-dominated responses is associated with humoral immunity and protection from autoimmune pathology. Overproduction of Th2-cytokines by allergen-specific $CD4^+$ T cells can result in the development of allergic disease and asthma, while Th1 cells have been associated with a variety of pro-inflammatory diseases.

One approach to immune associated diseases is immunotherapy. Immunotherapy has proven to be effective when used properly, and it is hoped that advances in immunologic intervention will further improve the efficacy. Alternative approaches have attempted to use cytokines to shift the immune response. IL-12, a heterodimeric cytokine produced by macrophages and dendritic cells, is potent in driving the development of Th1 cytokine synthesis in naive and memory $CD4^+$ T cells. Other cytokines, such as IL-13 and IL-4, have been associated with the differentiation of T cells to a Th2 type.

Atopy, which includes asthma, allergic rhinitis, and atopic dermatitis, is a complex trait that arises as a result of environmentally induced immune responses in genetically susceptible individuals. The prevalence of all atopic diseases has dramatically increased in industrialized countries over the past two decades. Asthma is the most common chronic disease of childhood and affects more than 15 million individuals in the United States, leading to direct treatment costs exceeding $11 billion per annum. Epidemiological studies have suggested that the increase in asthma prevalence results

from changes in hygiene and from reduced frequency of infections (e.g., tuberculosis or hepatitis A) within industrialized society. However, the specific molecular pathways that result in the increased asthma prevalence, and the genetic polymorphisms that confer asthma susceptibility are poorly understood.

Expression of asthma is influenced by multiple environmental and genetic factors that interact with each other in non-additive ways, complicating the identification of asthma susceptibility genes. Asthma susceptibility has been linked to several chromosomal regions, but with resolution no better than 5-10 cM, in which there are usually hundreds of candidate genes. Moreover, because the effects of genetic variation in any single gene are likely to have only modest effects in the overall pathogenesis of asthma, and because gene-gene and gene-environment interactions confound the analysis, the location of putative susceptibility genes to regions amenable to positional cloning has proven difficult to refine. Nevertheless, asthma susceptibility has been linked to chromosomes 5, 6, 11, 14, and 12. Of these, chromosome 5q23-35 has received the greatest attention because it contains a large number of candidate genes(11, 12, 13, 14, 15, 16, 17, 18), including IL-9, IL-12p40, the β-adrenergic receptor, and the IL-4 cytokine cluster, which contains the genes for IL-4, IL-5, and IL-13. However, the large size of the linked region of 5q complicates its analysis, and a gene for asthma from this site has not yet been conclusively identified.

Related Publications

The genetic sequence of the human hepatitis virus A cellular receptor may be found in Genbank, accession number XM_011327. A related sequence is provided in Genbank, accession number BAB55044. Monney *et al.* (2002) Nature 415:436 describe cell surface molecules expressed on Th1 cells. U.S. Patent nos. 5721351, US 6204371, US 6288218 relate to sequences corresponding to a mouse TIM-3 allele.

SUMMARY OF THE INVENTION

Genetic sequences of a gene family encoding polypeptides associated with immune function and cell survival are provided. These genes encode cell surface molecules with conserved IgV and mucin domains, herein referred to as T cell Immunoglobulin domain and Mucin domain (TIM) proteins. The locus comprising the TIM family is genetically associated with immune dysfunction, including asthma. Furthermore, the TIM gene family is located within a region of human chromosome 5 that is commonly deleted in malignancies and myelodysplastic syndrome. Polymorphisms are identified in TIM-1, TIM-3 and TIM-4, which can be associated with Th1/Th2 differentiation and airway hyperresponsiveness (AHR).

The nucleic acid compositions are used to produce the encoded proteins, which may be employed for functional studies, as a therapeutic, and in studying associated physiological pathways. TIM specific binding agents, including nucleic acids, antibodies, and the like, are useful as diagnostics for determining genetic susceptibility to atopy and asthma and as diagnostics for assessing tumor resistance to cancer therapy. TIM blocking agents find use as therapeutics in the treatment of immune dysfunction and disorders of cell survival, including malignancies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1*a*, *HBA mice produce significantly less IL-4 than do BALB/c mice.* Lymph node cells of mice immunized with 150 μg of KLH were harvested, B-cell depleted, and cultured in cDME with 10μg/ml KLH. Supernatants were harvested after 96 hours and assessed for IL-4 levels by ELISA. Shown is a box plot of IL-4 levels (n=10 in each group), representing the full range of data, with the boxes encompassing the upper and lower quartiles, with the median of the data set shown inside each box. IL-4 levels produced by BALB/c cells and by (BALB/c x HBA) F1 cells are significantly higher than HBA IL-4 levels P<0.0001 (student's t-test). *b, HBA mice produce significantly less IL-13 and IL-10 than do BALB/c mice.* Data shown are the average values of cytokines produced by lymph node cell cultures from ten individual mice in each experimental group, ± S.D. HBA IL-13 and IL-10 levels were lower than either the BALB/c or (BALB/c x HBA) F1 values, P<0.0001. HBA IL-5 versus BALB/c, P<0.05. HBA IFN-γ versus BALB/c, P<0.001. *c, Allergen-induced airway hyperreactivity is significantly greater in BALB/c than do HBA mice.* Pulmonary airflow obstruction was measured, and data shown represent peak enhanced pause (Penh) values averaged among sensitized mice in each group at various methacholine concentrations, ± S.E.M. (BALB x HBA) F1 demonstrate BALB/c phenotypes, while (BALB x DBA) F1 mice demonstrate DBA/2-like phenotypes.

Figure 2. Regions of HBA chromosome 11 were inherited from DBA/2. HBA chromosome 11 contains two regions derived from DBA between *hba-a2* and *es-3*, as delineated by SSLP markers. The regions of HBA chromosome 11 with DBA/2 genotypes are highlighted (in blue) in the diagram to the left. The markers (left) provide 2-5 cM resolution distal to *D11Mit230*. Where the mouse chromosome 11 has regions of highly conserved synteny with 5q23-35, additional markers were identified with informative polymorphisms between BALB/c and DBA/2, to provide 0.01-2 cM resolution in this region (right column markers). Single-stranded confirmation polymorphisms (SSCP) markers are shown in green to distinguish them from the SSLP markers, and the positions of particular genes of interest, denoted in red, are also shown. Where the arrangement of our marker map differs from the Chromosome Committee Reports and the MIT linkage map, our map

concurs with previous linkage and physical maps.

Figure 3a. *IL-4 production by N2 mice is bimodal, with peaks corresponding to F1 and HBA phenotypes.* As a means of evaluating the relative phenotypes of recombinant N2 mice in multiple experiments, we utilized an indexing function that allowed us to consolidate data from multiple experiments. A histogram shows a bimodal distribution of IL-4 index values for N2 mice with (BALB x HBA) F1, HBA homozygous, and recombinant haplotypes indicated. Distributions associated with the F1 and HBA haplotypes are distinct ($P<0.0001$, paired Student's t-test). *b. IL-4 regulation segregates with Kim1sscp.* Recombinant N2 haplotypes were sorted by IL-4 index values into groups associated with high IL-4 phenotypes (index < 0.35) and low IL-4 phenotypes (index > 0.65). Each column of boxes represents a recombinant haplotype. Alleles for these haplotypes at loci between D11Mit269 to D11Mit154 are shaded according to genotype (dark = F1; pale = HBA). High IL-4 production segregates with four haplotypes (left), and low IL-4 production segregates with four haplotypes (right). The IL-4 phenotype is linked to *Kim1sscp. c. AHR of N2 mice is bimodal, with peaks corresponding to F1 and HBA phenotypes.* Index values calculated from Penh values are shown. The histogram shows a bimodal distribution of AHR index values for N2 mice. (BALB x HBA) F1, HBA homozygous, and recombinant haplotypes are indicated. Distributions associated with the F1 and HBA haplotypes are distinct ($P<0.0001$, paired student's t-test). *d. AHR Regulatory Locus cosegregates with the IL-4 Regulatory Locus between D11Mit22 and D11Mit271.* Recombinant N2 haplotypes were sorted by AHR index values into groups associated with high AHR phenotypes (index < 0.35) and low AHR phenotypes (index > 0.65). Each column of boxes represents a recombinant haplotype. Alleles for these haplotypes at loci between D11Mit269 to D11Mit154 are shaded according to genotype (dark = F1; pale = HBA). High AHR segregates with four haplotypes (left), and low IL-4 production segregates with three haplotypes (right). The AHR regulatory locus is linked to *Kim1sscp,* between *D11Mit271* and *D11Mit22.*

Figure 4 *Mouse chromosome 11 interval containing Tapr is highly homologous to 5q33.* In order to construct a composite map around the *Tapr* locus, we integrated available information from the mouse linkage, backcross, and radiation hybrid maps and identified a region of highly conserved synteny in current maps of the human genome (Human Genome Browser v3, UCSC, March 2001). ESTs located on a physical map of mouse chromosome 11 (left) are denoted by their accession numbers and aligned by homology to genes (center), which correspond to ESTs on human chromosome 5 (right). All known genes between *KIAA0171* and *Sgd* are shown.

Figure 5a, b, c. *Identification novel TIM gene family and major polymorphisms in TIM-1 and TIM-3.* Cloning of mouse TIM-1 and mouse TIM-2. Members of a gene family.

Sequences of the mouse TIM gene family members are shown. Shaded boxes illustrate identity between two of the mouse TIM genes. Total RNA from conA-stimulated splenocytes was reverse transcribed using Gibco Superscript II. PCR products of full length *Tim-3* cDNA were amplified, purified with Qiagen QIAquick PCR Purification reagents, and sequenced directly by Biotech Core (Mountain View, CA). PCR products for *Tim-1* and *Tim-2* cDNA were cloned into electrocompetent TOP10 cells with TOPO-XL cloning reagents (Invitrogen). Plasmids were purifiifed with a standard alkaline lysis protocol. BALB/c and HBA plasmids were sequenced, as described. Homology of mouse TIM-1, rat KIM-1, and HAVcr-1. Identity with mouse TIM-1 is denoted by the shaded boxes. The approximate signal site is denoted by an open, inverted triangle and the Ig domain/ mucin domain boundary is shown with a filled diamond. The predicted transmembrane domains are underlined. TIM-1 and TIM-3 sequences with major polymorphisms between BALB/c and HBA TIM-1 and TIM-3 shown.

Figure 6. *Tapr Regulates CD4 T cell IL-4 and IL-13 Responses.* T cells from BALB/c DO11.10 mice produce higher levels of IL-4 and IL-13 in response to antigen than do T cells from HBA DO11.10 mice. Splenic CD4+ cells were isolated by positive selection with anti-CD4 magnetic beads and then cocultured with bone marrow-derived DCs and OVA. After seven days, the cells were restimulated. Supernatants were harvested after 18-24 hours of the secondary culture. Data represent mean cytokine levels detected at increasing concentrations of OVA, ±S.D. Detection of expression of TIM-1 mRNA in purified CD 4 T cells during priming and differentiation.

Figure 7. Sequence alignment of Human and Mouse TIM protein sequences.

Figure 8. Polymorphisms in human TIM-1

Figure 9. SSCP polymorphism analysis of human TIM-1.

DETAILED DESCRIPTION OF THE EMBODIMENTS

Genetic sequences associated with immune function, including susceptibility to asthma, are provided. The sequences of murine Tim-1, Tim-2, Tim-3, and Tim-4, and the human counterpart sequences, are provided herein. The sequence of major polymorphisms are also provided. Genomic sequences demonstrate that these polymorphisms, including a deletion, are true polymorphisms, not splicing variants. Variants of the TIM-1 and TIM-3 sequences are associated with susceptibility to airway hyperreactivity and allergic T cell responses, and other variants associated with protection against these responses.

The extracellular domain of TIM gene family members contains two domains, an IgV domain and a mucin domain. This Ig/mucin structure is also found in

MAdCAM (mucosal addressin cell adhesion molecule), which contains two Ig domains and a mucin domain; however, there is only a low level of homology between TIMs and MAdCAM. A similar degree of homology is also present between TIM-1 and TOSO (NP_005440.1 GI:4885641), a protein which, like TIM-1 is expressed on activated T cells, and which protects T cells from Fas-mediated apoptosis.

T cells express the TIM family of genes, which critically regulates CD4 T cell differentiation. Th1 cells preferentially express the TIM-3 protein, while Th2 cells preferentially express the TIM-1 protein. TIM-1 has been linked to airway hyperreactivity and TIM-3 to autoimmune disease, therefore the expression pattern on differentiating lymphoid cells and the kinetics of expression of TIM-1 on lymphoid cells reflect the function of these molecules.

In another aspect of the invention, methods are provided for determining susceptibility of an individual to developing asthma and atopic diseases, by determining the genotype at the Tim locus. Screening may analyze, for example, polymorphisms in any one of the TIM-1, TIM-3 or TIM-4 alleles provided herein, or otherwise determined. Methods are provided for screening such polymorphisms, *e.g.* SSCP analysis, size polymorphisms, and the like.

In another aspect of the invention, a method of screening for biologically active agents that modulate Tim gene or polypeptide function is provided, where the method comprises combining a candidate biologically active agent with any one of: (a) a Tim polypeptide; (b) a cell comprising a nucleic acid encoding a Tim polypeptide; or (c) a non-human transgenic animal model for Tim gene function comprising one of: (i) a knockout of an *Tim* gene; (ii) an exogenous and stably transmitted *Tim* gene sequence; or (iii) a *Tim* promoter sequence operably linked to a reporter gene; and determining the effect of said agent on Tim function.

The activity of TIM polypeptides may be modulated in order to direct immune function. TIM-1 is preferentially expressed in Th2 cells, and agents that modulate TIM-1 activity find use in the treatment of Th2 related disorders, including allergies, asthma, and the like. TIM-3 is preferentially expressed in Th1 cells, and agents that modulate TIM-3 activity find use in the treatment of pro-inflammatory immune diseases, including autoimmune diseases, graft rejection and the like.

CONDITIONS OF INTEREST

Atopic diseases are complex genetic traits that develop as a result of environmentally induced immune responses in genetically predisposed individuals. Both atopic and non-atopic individuals are exposed to the same environmental factors, but genetic differences that distinguish atopic from non-atopic individuals result in atopic

disease in some individuals, manifested by allergic inflammation in the respiratory tract, skin or gastrointestinal tract, as well as by elevated serum IgE, eosinophilia and the symptoms of wheezing, sneezing or hives. In addition, allergic inflammatory responses are characterized by the presence of Th2 lymphocytes producing high levels of IL-4, IL-5, IL-9 and IL-13, which enhance the growth, differentiation and/or recruitment of eosinophils, mast cells, basophils and B cells producing IgE.

Allergens of interest include antigens found in food, such as strawberries, peanuts, milk proteins, egg whites, *etc.* Other allergens of interest include various airborne antigens, such as grass pollens, animal danders, house mite feces, *etc.* Molecularly cloned allergens include *Dermatophagoides pteryonyssinus* (Der P1); Lol pI-V from rye grass pollen; a number of insect venoms, including venom from jumper ant *Myrmecia pilosula*; *Apis mellifera* bee venom phospholipase A2 (PLA_2 and antigen 5S; phospholipases from the yellow jacket *Vespula maculifrons* and white faced hornet *Dolichovespula maculata*; a large number of pollen proteins, including birch pollen, ragweed pollen, *Parol* (the major allergen of *Parietaria officinalis)* and the cross-reactive allergen *Parj*l (from *Parietaria judaica)*, and other atmospheric pollens including *Olea europaea*, *Artemisia sp.*, *gramineae*, *etc.* Other allergens of interest are those responsible for allergic dermatitis caused by blood sucking arthropods, *e.g. Diptera*, including mosquitos (*Anopheles sp.*, *Aedes sp.*, *Culiseta sp.*, *Culex sp.*); flies (*Phlebotomus sp.*, *Culicoides sp.*) particularly black flies, deer flies and biting midges; ticks (*Dermacenter sp.*, *Ornithodoros sp.*, *Otobius sp.*); fleas, *e.g.* the order *Siphonaptera*, including the genera *Xenopsylla*, *Pulex* and *Ctenocephalides felis felis*. The specific allergen may be a polysaccharide, fatty acid moiety, protein, *etc.*

Polymorphisms in a number of interacting/epistatic atopy genes are thought to result in enhanced susceptibility to allergic disorders. Genome wide scans have sought to identify the responsible genes by linking various parameters of allergy and asthma with polymorphic DNA markers of specific genes, usually repeat sequences of DNA (microsatellites that contain di- tri- and tetra-nucleotide repeats). These studies have identified several chromosomal regions as likely to be involved in the pathogenesis of atopy, but with resolution no better than 5-10 cM in which there are typically hundreds of candidate genes. Nevertheless, asthma susceptibility has been linked to chromosomes 5q23-31, chromosome 6p21, chromosome 11q13, and chromosome 12q (9-13) by two or more of these genome wide scanning studies.

Asthma, as defined herein, is reversible airflow limitation in an individual over a period of time. Asthma is characterized by the presence of cells such as eosinophils, mast cells, basophils, and $CD25^+$ T lymphocytes in the airway walls. There is a close interaction between these cells, because of the activity of cytokines which have a variety of communication and biological effector properties. Chemokines attract cells to the site of

inflammation and cytokines activate them, resulting in inflammation and damage to the mucosa. With chronicity of the process, secondary changes occur, such as thickening of basement membranes and fibrosis. The disease is characterized by increased airway hyperresponsiveness to a variety of stimuli, and airway inflammation. A patient diagnosed as asthmatic will generally have multiple indications over time, including wheezing, asthmatic attacks, and a positive response to methacholine challenge, i.e., a PC20 on methacholine challenge of less than about 4 mg/ml. Guidelines for diagnosis may be found, for example, in the National Asthma Education Program Expert Panel Guidelines for Diagnosis and Management of Asthma, National Institutes of Health, 1991, Pub. No. 91-3042.

Asthma, allergic rhinitis (hay fever), atopic dermatitis (eczema) and food allergy are diseases that occur in the same families, implying common genetic mechanisms. These atopic diseases are exceedingly prevalent, affecting 20-40% of the general population and constitute a major public health problem. The economic costs for these disorders are enormous. For asthma alone, the estimated health care costs in 1996 were $14 billion. In addition, the prevalence of all of the atopic diseases has increased dramatically in industrialized countries over the past two decades for reasons that are not yet clear. The prevalence of asthma in industrialized countries, for which the numbers are the most accurate, has doubled since 1982, and is projected to double again in prevalence by the year 2020.

Pro-inflammatory diseases associated with Th1 type T cells include autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, type I diabetes mellitus, *etc.* (RA) is a chronic autoimmune inflammatory synovitis affecting 0.8% of the world population. Current therapy for RA utilizes therapeutic agents that non-specifically suppress or modulate immune function. Such therapeutics, including the recently developed TNFα antagonists, are not fundamentally curative, and disease activity rapidly returns following discontinuation of therapy. Tremendous clinical need exists for fundamentally curative therapies that do not cause systemic immune suppression or modulation.

A quantitative increase in myelin-autoreactive T cells with the capacity to secrete IFN-gamma is associated with the pathogenesis of MS and EAE, suggesting that autoimmune inducer/helper T lymphocytes in the peripheral blood of MS patients may initiate and/or regulate the demyelination process in patients with MS. The overt disease is associated with muscle weakness, loss of abdominal reflexes, visual defects and paresthesias. During the presymptomatic period there is infiltration of leukocytes into the cerebrospinal fluid, inflammation and demyelination.

IDDM is a cell-mediated autoimmune disorder leading to destruction of insulin-secreting beta cells and overt hyperglycemia. T lymphocytes invade the islets of Langerhans, and specifically destroy insulin-producing β-cells. The depletion of β cells results in an inability to regulate levels of glucose in the blood. The disease progression may be monitored in individuals diagnosed by family history and genetic analysis as being susceptible. The most important genetic effect is seen with genes of the major histocompatibility locus (*IDDM1*), although other loci, including the insulin gene region (*IDDM2*) also show linkage to the disease (see Davies *et al, supra* and Kennedy *et al.* (1995) Nature Genetics 9:293-298).

TIM GENE FAMILY

The provided TIM family genes and fragments thereof, encoded proteins, genomic regulatory regions, and specific antibodies are useful in the identification of individuals predisposed to development or resistance to asthma, and for the modulation of gene activity in vivo for prophylactic and therapeutic purposes. The encoded proteins are useful as an immunogen to raise specific antibodies, in drug screening for compositions that mimic or modulate activity or expression, including altered forms of the proteins, and as a therapeutic.

The TIM family genes are immediately adjacent to each other on human chromosome 5, in the order TIM-4, TIM-1, TIM-3, with no intervening genes. This segment of human chromosome 5 is commonly deleted in malignancies and dysplastic cell populations, as in myeolodysplastic syndrome (see Boultwood, *et al*, (1997) Genomics 45:88-96). There are TIM pseudogenes on chromosomes 5, 12, and 19. Each TIM protein, except TIM-4, contains a distinct predicted tyrosine signaling motif. The cytoplasmic region of TIM-1 contains two tyrosine residues and includes a highly conserved tyrosine kinase phosphorylation motif, RAEDNIY. The expanded region, SRAEDNIYIVEDRP, contains a predicted site for Itk phosphorylation and for EGF-receptor phosphorylation.

The mouse *Tim1* gene encodes a 305 amino acid membrane protein. The cytoplasmic region of TIM-1 contains two tyrosine residues and includes a highly conserved tyrosine kinase phosphorylation motif, RAEDNIY. The mucin domain of TIM-1 has multiple sites for O-linked glycosylation, and there two sites for N-linked glycosylation found in the immunoglobulin domain.

Mouse TIM-2 , a similar 305 amino acid membrane protein, has 64% identity to mouse TIM-1, 60% identity to rat KIM-1, and 32% identity to hHAVcr-1. Like TIM-1, TIM-2 has two extracellular N-linked glycosylation sites and a serine, threonine- rich mucin domain with many O-linked glycosylation sites. TIM-2 also has an intracellular tyrosine

kinase phosphorylation motif, RTRCEDQVY.

Tim3 encodes a 281 amino acid membrane protein in mice, and a 301 amino acid protein in humans, that has a similar, integral membrane glycoprotein structure with multiple extracellular glycosylation sites and an intracellular tyrosine phosphorylation motif. Although the mucin domain is not as prominent in TIM-3 as it is in TIM-1 and TIM-2, TIM-3 expressed on T cells interacts with a ligand on APCs and alters APC activation. TIM-3 has four sites for N-linked and five sites for O-linked glycosylation, suggesting that TIM-3, like TIM-1 and TIM-2, is heavily glycosylated and might interact with a ligand present on other cells, such as antigen presenting cells.

Tim4 encodes a 344 amino acid protein in mice, and a 378 amino acid protein in humans. The predicted TIM-4 also shares the general membrane glycoprotein structural motifs of the other TIM proteins, a with an IgV-like domain with highly conserved cysteine residues, a threonine-rich mucin-like domain, and a short intracellular tail.

Polymorphisms in the murine sequences are provided in the sequence listing for the BALB/c and HBA/DBA strains. In TIM-1, these polymorphisms encode three amino acid differences and a fifteen amino acid deletion in HBA/DBA. Seven predicted amino acid differences were identified in TIM-3. The polymorphisms in TIM-1 and TIM-4 are located in the signal and mucin-like domains, while the polymorphisms identified in TIM-3 are clustered in the Ig domain.

Variants in coding regions of human *Tim1* are provided in the seqlist and Figure 8. Variations include an insertion (labeled polymorphism 1), 157insMTTTVP, observed in 65% of the chromosomes, and a deletion (polymorphism 5), 187ΔThr, observed in 65% of the chromosomes. Other polymorphisms are T140A (polymorphism 7); V161A; (polymorphism 2); V167I (polymorphism 3); T172A (polymorphism 4); N258D (polypmorphism 6). Polymorphism 4 was observed in 40% of the chromosomes, and the other polymorphisms were each observed in ≤5% of the chromosomes. Most of these variations (2-6) are located within exon 3, the first mucin-encoding exon, and all of the variants occur at the genomic level and are not splice variants. The association between *Tim1* and asthma susceptibility is further supported by reports of significant linkage of mite-sensitive childhood asthma to D5S820 (mean LOD score = 4.8), a marker which is approximately 0.5 megabases from *Tim1*.

In human tissues, a 4.4 kb TIM-1 mRNA is present in almost all tissues, though it is faint in most. A 5.5-kb band was observed in colon and liver. A 7.5-kb band was observed in spleen, thymus, and peripheral blood leukocytes, and smaller than 4.4-kb bands were observed in some organs. TIM-1 mRNA is expressed with alternate 5' untranslated regions, in different cell populations. Hypoxia and ischemia induces TIM-1 expression in epithelial cells, and radiation induces expression of TIM gene family mRNA.

The TIM genes are expressed in tumor specimens. Human TIM-4 mRNA is expressed in glioblastoma tissue, and is also detected in mitogen stimulated or irradiated peripheral blood monocytes.

In one aspect, the invention provides for an isolated nucleic acid molecule other than a naturally occurring chromosome comprising a sequence encoding a TIM-1, TIM-2, TIM-3 or TIM-4 protein, or a homolog or variant thereof, which variant may be associated with susceptibility to airway hyperreactivity and allergic T cell responses. The nucleic acid may be operably linked to a vector and/or control sequences for expression in a homologous or heterologous host cell. Such a host cell can find use in the production of the encoded protein. In another aspect of the invention, a purified polypeptide is provided of TIM-1, TIM-2, TIM-3 or TIM-4 protein, or a homolog or variant thereof, which variant may be associated with susceptibility to airway hyperreactivity and allergic T cell responses. In another aspect, an antibody or other specific binding member that binds to the TIM-1, TIM-2, TIM-3 or TIM-4 polypeptide is provided.

Sequences of human and murine TIM sequences are provided in the sequence listing, as follows:

SEQ ID NO	Name	Length	Type
1	BALB/c TIM-1	305	Protein
2	BALB/c TIM-1	918	DNA
3	ES-HBA TIM-1	282	Protein
4	ES-HBA TIM-1	849	DNA
5	BALB/c TIM-2	305	Protein
6	BALB/c TIM-1	958	DNA
7	ES-HBA TIM-2	305	Protein
8	ES-HBA TIM-2	958	DNA
9	BALB/c TIM-3	281	Protein
10	BALB/c TIM-3	2725	DNA
11	ES-HBA TIM-3	281	Protein
12	ES-HBA TIM-3	862	DNA
13	BALB/c TIM-4	345	Protein
14	BALB/c TIM-4	1032	DNA
15	ES-HBA TIM-4	345	Protein
16	ES-HBA TIM-4	1032	DNA
17	hTIM-1 allele 1	359	Protein
18	hTIM-1 allele 1	1080	DNA
19	hTIM-1 allele 2	359	Protein
20	hTIM-1 allele 2	1080	DNA
21	hTIM-1 allele 3	365	Protein
22	hTIM-1 allele 3	1098	DNA
23	hTIM-1 allele 4	359	Protein
24	hTIM-1 allele 4	1079	DNA
25	hTIM-1 allele 5	364	Protein
26	hTIM-1 allele 5	1095	DNA
27	hTIM-1 allele 6	364	Protein
28	hTIM-1 allele 6	1099	DNA
29	hTIM-3 allele 1	301	Protein
30	hTIM-3 allele 1	1116	DNA

31	hTIM-3 allele 2	301	Protein
32	hTIM-3 allele 2	1116	DNA
33	hTIM-4 allele 1	378	Protein
34	hTIM-4 allele 1	1156	DNA
35	hTIM-4 allele 2	378	Protein
36	hTIM-4 allele 2	1156	DNA

The DNA sequence encoding Tim polypeptides may be cDNA or genomic DNA or a fragment thereof. Fragments of interest for probes, producing polypeptides, *etc.* may comprise one or more polymorphic residues. The term Tim gene shall be intended to mean the open reading frame encoding any one of the specific Tim polypeptides, introns, as well as adjacent 5' and 3' non-coding nucleotide sequences involved in the regulation of expression, up to about 1 kb beyond the coding region, but possibly further in either direction. The gene may be introduced into an appropriate vector for extrachromosomal maintenance or for integration into the host.

In some embodiments, the Tim gene sequence is other than human TIM-1 allele 1, as set forth in the sequence listing; and/or other than mouse TIM-3 DBA allele.

The term "cDNA" as used herein is intended to include all nucleic acids that share the arrangement of sequence elements found in native mature mRNA species, where sequence elements are exons and 3' and 5' non-coding regions. Normally mRNA species have contiguous exons, with the intervening introns removed by nuclear RNA splicing, to create a continuous open reading frame encoding a Tim protein.

A genomic sequence of interest comprises the nucleic acid present between the initiation codon and the stop codon, as defined in the listed sequences, including all of the introns that are normally present in a native chromosome. It may further include the 3' and 5' untranslated regions found in the mature mRNA. It may further include specific transcriptional and translational regulatory sequences, such as promoters, enhancers, *etc.*, including about 1 kb, but possibly more, of flanking genomic DNA at either the 5' or 3' end of the transcribed region. The genomic DNA may be isolated as a fragment of 100 kbp or smaller; and substantially free of flanking chromosomal sequence.

The sequence of the 5' region, and further 5' upstream sequences and 3' downstream sequences, may be utilized for promoter elements, including enhancer binding sites, that provide for expression in tissues where *Tim* genes are expressed. The tissue specific expression is useful for determining the pattern of expression, and for providing promoters that mimic the native pattern of expression. Naturally occurring polymorphisms in the promoter region are useful for determining natural variations in expression, particularly those that may be associated with disease. Alternatively, mutations may be introduced into the promoter region to determine the effect of altering expression in experimentally defined systems. Methods for the identification of specific DNA motifs

involved in the binding of transcriptional factors are known in the art, *e.g.* sequence similarity to known binding motifs, gel retardation studies, *etc.* For examples, see Blackwell *et al.* (1995) Mol Med **1**: 194-205; Mortlock *et al.* (1996) Genome Res. **6**: 327-33; and Joulin and Richard-Foy (1995) Eur J Biochem **232**: 620-626.

The regulatory sequences may be used to identify *cis* acting sequences required for transcriptional or translational regulation of TIM expression, especially in different tissues or stages of development, and to identify *cis* acting sequences and *trans* acting factors that regulate or mediate TIM expression. Such transcription or translational control regions may be operably linked to a TIM gene in order to promote expression of wild type or altered TIM or other proteins of interest in cultured cells, or in embryonic, fetal or adult tissues, and for gene therapy.

The nucleic acid compositions of the subject invention may encode all or a part of the subject polypeptides. Fragments may be obtained of the DNA sequence by chemically synthesizing oligonucleotides in accordance with conventional methods, by restriction enzyme digestion, by PCR amplification, *etc.* For the most part, DNA fragments will be of at least 15 nt, usually at least 18 nt, more usually at least about 50 nt. Such small DNA fragments are useful as primers for PCR, hybridization screening, *etc.* Larger DNA fragments, *i.e.* greater than 100 nt are useful for production of the encoded polypeptide. For use in amplification reactions, such as PCR, a pair of primers will be used. The exact composition of the primer sequences is not critical to the invention, but for most applications the primers will hybridize to the subject sequence under stringent conditions, as known in the art. It is preferable to choose a pair of primers that will generate an amplification product of at least about 50 nt, preferably at least about 100 nt. Algorithms for the selection of primer sequences are generally known, and are available in commercial software packages. Amplification primers hybridize to complementary strands of DNA, and will prime towards each other.

The *TIM* genes are isolated and obtained in substantial purity, generally as other than an intact mammalian chromosome. Usually, the DNA will be obtained substantially free of other nucleic acid sequences that do not include an *TIM* sequence or fragment thereof, generally being at least about 50%, usually at least about 90% pure and are typically "recombinant", *i.e.* flanked by one or more nucleotides with which it is not normally associated on a naturally occurring chromosome.

The DNA sequences are used in a variety of ways. They may be used as probes for identifying *TIM* related genes. Mammalian homologs have substantial sequence similarity to the subject sequences, *i.e.* at least 75%, usually at least 90%, more usually at least 95% sequence identity with the nucleotide sequence of the subject DNA sequence. Sequence similarity is calculated based on a reference sequence, which may be a subset

of a larger sequence, such as a conserved motif, coding region, flanking region, *etc.* A reference sequence will usually be at least about 18 nt long, more usually at least about 30 nt long, and may extend to the complete sequence that is being compared. Algorithms for sequence analysis are known in the art, such as BLAST, described in Altschul *et al.* (1990) J Mol Biol **215**:403-10.

Nucleic acids having sequence similarity are detected by hybridization under low stringency conditions, for example, at 50°C and 10XSSC (0.9 M saline/0.09 M sodium citrate) and remain bound when subjected to washing at 55°C in 1XSSC. Sequence identity may be determined by hybridization under stringent conditions, for example, at 50°C or higher and 0.1XSSC (9 mM saline/0.9 mM sodium citrate). By using probes, particularly labeled probes of DNA sequences, one can isolate homologous or related genes. The source of homologous genes may be any species, *e.g.* primate species, particularly human; rodents, such as rats and mice, canines, felines, bovines, ovines, equines, yeast, *Drosophila*, *Caenhorabditis*, *etc.*

The DNA may also be used to identify expression of the gene in a biological specimen. The manner in which one probes cells for the presence of particular nucleotide sequences, as genomic DNA or RNA, is well established in the literature and does not require elaboration here. mRNA is isolated from a cell sample. mRNA may be amplified by RT-PCR, using reverse transcriptase to form a complementary DNA strand, followed by polymerase chain reaction amplification using primers specific for the subject DNA sequences. Alternatively, mRNA sample is separated by gel electrophoresis, transferred to a suitable support, *e.g.* nitrocellulose, nylon, *etc.*, and then probed with a fragment of the subject DNA as a probe. Other techniques, such as oligonucleotide ligation assays, *in situ* hybridizations, and hybridization to DNA probes arrayed on a solid chip may also find use. Detection of mRNA hybridizing to the subject sequence is indicative of *TIM* gene expression in the sample.

The subject nucleic acid sequences may be modified for a number of purposes, particularly where they will be used intracellularly, for example, by being joined to a nucleic acid cleaving agent, *e.g.* a chelated metal ion, such as iron or chromium for cleavage of the gene; or the like.

The sequence of the *TIM* locus, including flanking promoter regions and coding regions, may be mutated in various ways known in the art to generate targeted changes in promoter strength, sequence of the encoded protein, *etc.* The DNA sequence or product of such a mutation will be substantially similar to the sequences provided herein, *i.e.* will differ by at least one nucleotide or amino acid, respectively, and may differ by at least two but not more than about ten nucleotides or amino acids. The sequence changes may be substitutions, insertions or deletions. Deletions may further include larger changes,

such as deletions of a domain or exon. Other modifications of interest include epitope tagging, *e.g.* with the FLAG system, HA, *etc.* For studies of subcellular localization, fusion proteins with green fluorescent proteins (GFP) may be used. Such mutated genes may be used to study structure-function relationships of TIM polypeptides, or to alter properties of the protein that affect its function or regulation. For example, constitutively active transcription factors, or a dominant negatively active protein that binds to the TIM DNA target site without activating transcription, may be created in this manner.

Techniques for *in vitro* mutagenesis of cloned genes are known. Examples of protocols for scanning mutations may be found in Gustin *et al.*, *Biotechniques* 14:22 (1993); Barany, *Gene* 37:111-23 (1985); Colicelli *et al.*, *Mol Gen Genet* 199:537-9 (1985); and Prentki *et al.*, *Gene* 29:303-13 (1984). Methods for site specific mutagenesis can be found in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, CSH Press 1989, pp. 15.3-15.108; Weiner *et al.*, *Gene* 126:35-41 (1993); Sayers *et al.*, *Biotechniques* 13:592-6 (1992); Jones and Winistorfer, *Biotechniques* 12:528-30 (1992); Barton *et al.*, *Nucleic Acids Res* 18:7349-55 (1990); Marotti and Tomich, *Gene Anal Tech* 6:67-70 (1989); and *Zhu Anal Biochem* 177:120-4 (1989).

Arrays provide a high throughput technique that can assay a large number of polynucleotides in a sample. In one aspect of the invention, an array is constructed comprising one or more of the TIM genes, proteins or antibodies, preferably comprising all of these sequences, which array may further comprise other sequences known to be up- or down-regulated in T cells, monocytes, and the like. This technology can be used as a tool to test for differential expression, or for genotyping. Arrays can be created by spotting polynucleotide probes onto a substrate (*e.g.*, glass, nitrocellulose, *etc.*) in a two-dimensional matrix or array having bound probes. The probes can be bound to the substrate by either covalent bonds or by non-specific interactions, such as hydrophobic interactions. Techniques for constructing arrays and methods of using these arrays are described in, for example, Schena *et al.* (1996) Proc Natl Acad Sci U S A. **93**(20):10614-9; Schena *et al.* (1995) Science **270**(5235):467-70; Shalon *et al.* (1996) Genome Res. **6**(7):639-45, USPN 5,807,522, EP 799 897; WO 97/29212; WO 97/27317; EP 785 280; WO 97/02357; USPN 5,593,839; USPN 5,578,832; EP 728 520; USPN 5,599,695; EP 721 016; USPN 5,556,752; WO 95/22058; and USPN 5,631,734.

The probes utilized in the arrays can be of varying types and can include, for example, synthesized probes of relatively short length (e.g., a 20-mer or a 25-mer), cDNA (full length or fragments of gene), amplified DNA, fragments of DNA (generated by restriction enzymes, for example) and reverse transcribed DNA. Both custom and generic arrays can be utilized in detecting differential expression levels. Custom arrays can be prepared using probes that hybridize to particular preselected subsequences of mRNA

gene sequences or amplification products prepared from them.

Arrays can be used to, for example, examine differential expression of genes and can be used to determine gene function. For example, arrays can be used to detect differential expression, or expression of polymorphic sequences, in TIM genes. Exemplary uses of arrays are further described in, for example, Pappalarado *et al.* (1998) Sem. Radiation Oncol. **8**:217; and Ramsay. (1998) Nature Biotechnol. **16**:40. Furthermore, many variations on methods of detection using arrays are well within the skill in the art and within the scope of the present invention. For example, rather than immobilizing the probe to a solid support, the test sample can be immobilized on a solid support which is then contacted with the probe. Additional discussion regarding the use of microarrays in expression analysis can be found, for example, in Duggan, et al., Nature Genetics Supplement 21:10-14 (1999); Bowtell, Nature Genetics Supplement 21:25-32 (1999); Brown and Botstein, Nature Genetics Supplement 21:33-37 (1999); Cole et al., Nature Genetics Supplement 21:38-41 (1999); Debouck and Goodfellow, Nature Genetics Supplement 21:48-50 (1999); Bassett, Jr., et al., Nature Genetics Supplement 21:51-55 (1999); and Chakravarti, Nature Genetics Supplement 21:56-60 (1999).

Pharmacogenetics is the linkage between an individual's genotype and that individual's ability to metabolize or react to a therapeutic agent. Differences in metabolism or target sensitivity can lead to severe toxicity or therapeutic failure by altering the relation between bioactive dose and blood concentration of the drug. In the past few years, numerous studies have established good relationships between polymorphisms in metabolic enzymes or drug targets, and both response and toxicity. These relationships can be used to individualize therapeutic dose administration.

Genotyping of polymorphic alleles is used to evaluate whether an individual will respond well to a particular therapeutic regimen. The polymorphic sequences are also used in drug screening assays, to determine the dose and specificity of a candidate therapeutic agent. A candidate TIM polymorphism is screened with a target therapy to determine whether there is an influence on the effectiveness in treating asthma. Drug screening assays are performed as described above. Typically two or more different sequence polymorphisms are tested for response to a therapy.

The subject gene may be employed for synthesis of a complete TIM protein, or polypeptide fragments thereof, particularly fragments corresponding to functional domains; binding sites; *etc.*; and including fusions of the subject polypeptides to other proteins or parts thereof. For expression, an expression cassette may be employed, providing for a transcriptional and translational initiation region, which may be inducible or constitutive, where the coding region is operably linked under the transcriptional control of the transcriptional initiation region, and a transcriptional and translational termination

region. Various transcriptional initiation regions may be employed that are functional in the expression host.

Polypeptides of particular interest that are fragments of the TIM polypeptides include specific domains of the TIM polypeptides, where a domain may comprise, for example, the extracellular domain, or the domains within the extracellular domain: the mucin domain and/or the Ig domain. Domains may also comprise the cytoplasmic domain, *e.g.* a fragment encompassing the tyrosine kinase phosphorylation motif, RAEDNIY, or the expanded region, SRAEDNIYIVEDRP. Polypeptides encoded by the soluble splice variants are also of interest. The sequence of the Ig domains are as follows: human TIM-1 Ig domain, SEQ ID NO: 17, 19, 21, 23, 25, 27, residues 21-126; human TIM-3 Ig domain, SEQ ID NO: 29 and 31 , residues 22-131; human TIM-4 Ig domain, SEQ ID NO: 33 and 35, residues 25-133; mouse TIM-1 Ig domain, SEQ ID NO: 1 and 3, residues 21-129; mouse TIM-2 Ig domain, SEQ ID NO: 7, residues 22-128; mouse TIM-3 Ig domain, BALB/c allele, SEQ ID NO: 9, residues 22-132; mouse TIM-3 Ig domain, DBA/2 allele, SEQ ID No: 11, residues 22-132; mouse TIM-4 Ig domain, SEQ ID NO: 13 and 15, residues 25-135.

Functionally equivalent polypeptides may find use, where the equivalent polypeptide may contain deletions, additions or substitutions of amino acid residues that result in a silent change, thus producing a functionally equivalent differentially expressed on pathway gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. "Functionally equivalent", as used herein, refers to a protein capable of exhibiting a substantially similar *in vivo* activity as the polypeptide encoded by a TIM gene.

The polypeptides may be expressed in prokaryotes or eukaryotes in accordance with conventional ways, depending upon the purpose for expression. For large scale production of the protein, a unicellular organism, such as *E. coli, B. subtilis, S. cerevisiae,* or cells of a higher organism such as vertebrates, particularly mammals, *e.g.* COS 7 cells, may be used as the expression host cells. In many situations, it may be desirable to express the *TIM* gene in mammalian cells, where the *TIM* gene will benefit from native folding and post-translational modifications. Small peptides can also be synthesized in the laboratory, including specific peptide epitopes, domains, and the like, where peptides will usually be at least about 8 amino acids in length, more usually at least about 20 amino acids in length, up to complete domains, and the full length protein. Peptides may comprise polymorphic regions of the protein. Also included are fusion proteins, where all or a fragment of the TIM protein is fused to a heterologous polypeptide, e.g. green fluorescent protein, antibody Fc regions, poly-histidine, and the like.

In mammalian host cells, a number of viral-based expression systems may

be used, including retrovirus, lentivirus, adenovirus, adeno-associated virus, and the like. In cases where an adenovirus is used as an expression vector, the coding sequence of interest can be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing differentially expressed or pathway gene protein in infected hosts.

Specific initiation signals may also be required for efficient translation of the genes. These signals include the ATG initiation codon and adjacent sequences. In cases where a complete gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the gene coding sequence is inserted, exogenous translational control signals must be provided. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, *etc.*

With the availability of the polypeptides in large amounts, by employing an expression host, the polypeptides may be isolated and purified in accordance with conventional ways. A lysate may be prepared of the expression host and the lysate purified using HPLC, exclusion chromatography, gel electrophoresis, affinity chromatography, or other purification technique. The purified polypeptide will generally be at least about 80% pure, preferably at least about 90% pure, and may be up to and including 100% pure. Pure is intended to mean free of other proteins, as well as cellular debris.

The polypeptide may be labeled, either directly or indirectly. Any of a variety of suitable labeling systems may be used, including but not limited to, radioisotopes such as ^{125}I; enzyme labeling systems that generate a detectable colorimetric signal or light when exposed to substrate; and fluorescent labels. Indirect labeling involves the use of a protein, such as a labeled antibody, that specifically binds to the polypeptide of interest. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by a Fab expression library.

SPECIFIC BINDING MEMBERS

The term "specific binding member" or "binding member" as used herein refers to a member of a specific binding pair, i.e. two molecules, usually two different molecules, where one of the molecules (i.e., first specific binding member) through chemical or physical means specifically binds to the other molecule (i.e., second specific

binding member). The complementary members of a specific binding pair are sometimes referred to as a ligand and receptor; or receptor and counter-receptor. For the purposes of the present invention, the two binding members may be known to associate with each other, for example where an assay is directed at detecting compounds that interfere with the association of a known binding pair. Alternatively, candidate compounds suspected of being a binding partner to a compound of interest may be used.

Specific binding pairs of interest include carbohydrates and lectins; complementary nucleotide sequences; peptide ligands and receptor; effector and receptor molecules; hormones and hormone binding protein; enzyme cofactors and enzymes; enzyme inhibitors and enzymes; lipid and lipid-binding protein; etc. The specific binding pairs may include analogs, derivatives and fragments of the original specific binding member. For example, a receptor and ligand pair may include peptide fragments, chemically synthesized peptidomimetics, labeled protein, derivatized protein, etc.

In a preferred embodiment, the specific binding member is an antibody. The term "antibody" or "antibody moiety" is intended to include any polypeptide chain-containing molecular structure with a specific shape that fits to and recognizes an epitope, where one or more non-covalent binding interactions stabilize the complex between the molecular structure and the epitope. Antibodies that bind specifically to one of the TIM proteins are referred to as anti-TIM. The archetypal antibody molecule is the immunoglobulin, and all types of immunoglobulins, IgG, IgM, IgA, IgE, IgD, etc., from all sources, e.g. human, rodent, rabbit, cow, sheep, pig, dog, other mammal, chicken, other avians, etc., are considered to be "antibodies." Antibodies utilized in the present invention may be polyclonal antibodies, although monoclonal antibodies are preferred because they may be reproduced by cell culture or recombinantly, and can be modified to reduce their antigenicity.

Polyclonal antibodies can be raised by a standard protocol by injecting a production animal with an antigenic composition, which may be a polypeptide or a cDNA expressed *in vivo*. See, e.g., Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988. When utilizing an entire protein, or a larger section of the protein, antibodies may be raised by immunizing the production animal with the protein and a suitable adjuvant (e.g., Fruend's, Fruend's complete, oil-in-water emulsions, etc.) When a smaller peptide is utilized, it is advantageous to conjugate the peptide with a larger molecule to make an immunostimulatory conjugate. Commonly utilized conjugate proteins that are commercially available for such use include bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). In order to raise antibodies to particular epitopes, such as polymorphic residues, peptides derived from the full sequence may be utilized. The immunogen is injected into the animal host, preferably according to a predetermined

schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

Alternatively, for monoclonal antibodies, hybridomas may be formed by isolating the stimulated immune cells, such as those from the spleen of the inoculated animal. These cells are then fused to immortalized cells, such as myeloma cells or transformed cells, which are capable of replicating indefinitely in cell culture, thereby producing an immortal, immunoglobulin-secreting cell line. The immortal cell line utilized is preferably selected to be deficient in enzymes necessary for the utilization of certain nutrients. Many such cell lines (such as myelomas) are known to those skilled in the art, and include, for example: thymidine kinase (TK) or hypoxanthine-guanine phosphoriboxyl transferase (HGPRT). These deficiencies allow selection for fused cells according to their ability to grow on, for example, hypoxanthine aminopterinthymidine medium (HAT).

Preferably, the immortal fusion partners utilized are derived from a line that does not secrete immunoglobulin. The resulting fused cells, or hybridomas, are cultured under conditions that allow for the survival of fused, but not unfused, cells and the resulting colonies screened for the production of the desired monoclonal antibodies. Colonies producing such antibodies are cloned, expanded, and grown so as to produce large quantities of antibody, see Kohler and Milstein, 1975 Nature 256:495 (the disclosures of which are hereby incorporated by reference).

Large quantities of monoclonal antibodies from the secreting hybridomas may then be produced by injecting the clones into the peritoneal cavity of mice and harvesting the ascites fluid therefrom. The mice, preferably primed with pristane, or some other tumor-promoter, and immunosuppressed chemically or by irradiation, may be any of various suitable strains known to those in the art. The ascites fluid is harvested from the mice and the monoclonal antibody purified therefrom, for example, by CM Sepharose column or other chromatographic means. Alternatively, the hybridomas may be cultured *in vitro* or as suspension cultures. Batch, continuous culture, or other suitable culture processes may be utilized. Monoclonal antibodies are then recovered from the culture medium or supernatant.

In addition, the antibodies or antigen binding fragments may be produced by genetic engineering. In this technique, as with the standard hybridoma procedure, antibody-producing cells are sensitized to the desired antigen or immunogen. The messenger RNA isolated from the immune spleen cells or hybridomas is used as a template to make cDNA using PCR amplification. A library of vectors, each containing one heavy chain gene and one light chain gene retaining the initial antigen specificity, is produced by insertion of appropriate sections of the amplified immunoglobulin cDNA into

the expression vectors. A combinatorial library is constructed by combining the heavy chain gene library with the light chain gene library. This results in a library of clones which co-express a heavy and light chain (resembling the Fab fragment or antigen binding fragment of an antibody molecule). The vectors that carry these genes are co-transfected into a host (e.g. bacteria, insect cells, mammalian cells, or other suitable protein production host cell.). When antibody gene synthesis is induced in the transfected host, the heavy and light chain proteins self-assemble to produce active antibodies that can be detected by screening with the antigen or immunogen.

Chimeric antibodies may be made by recombinant means by combining the murine variable light and heavy chain regions (VK and VH), obtained from a murine (or other animal-derived) hybridoma clone, with the human constant light and heavy chain regions, in order to produce an antibody with predominantly human domains. The production of such chimeric antibodies is well known in the art, and may be achieved by standard means (as described, e.g., in U.S. Patent No. 5,624,659, incorporated fully herein by reference). Humanized antibodies are engineered to contain even more human-like immunoglobulin domains, and incorporate only the complementarity-determining regions of the animal-derived antibody. This is accomplished by carefully examining the sequence of the hyper-variable loops of the variable regions of the monoclonal antibody, and fitting them to the structure of the human antibody chains. Although facially complex, the process is straightforward in practice. See, e.g., U.S. Patent No. 6,187,287, incorporated fully herein by reference.

Alternatively, polyclonal or monoclonal antibodies may be produced from animals that have been genetically altered to produce human immunoglobulins. Techniques for generating such animals, and deriving antibodies therefrom, are described in U.S. Patents No. 6,162,963 and 6,150,584, incorporated fully herein by reference.

Alternatively, single chain antibodies (Fv, as described below) can be produced from phage libraries containing human variable regions. See U.S. Patent No. 6,174,708. Intrathecal administration of single-chain immunotoxin, LMB-7 [B3(Fv)- PE38], has been shown to cure of carcinomatous meningitis in a rat model. *Proc Natl. Acad. Sci U S A* 92, 2765-9, all of which are incorporated by reference fully herein.

In addition to entire immunoglobulins (or their recombinant counterparts), immunoglobulin fragments comprising the epitope binding site (e.g., Fab', $F(ab')_2$, or other fragments) are useful as antibody moieties in the present invention. Such antibody fragments may be generated from whole immunoglobulins by pepsin, papain, or other protease cleavage. "Fragment," or minimal immunoglobulins may be designed utilizing recombinant immunoglobulin techniques. For instance "Fv" immunoglobulins for use in the present invention may be produced by linking a variable light chain region to a variable

heavy chain region via a peptide linker (e.g., poly-glycine or another sequence which does not form an alpha helix or beta sheet motif).

Fv fragments are heterodimers of the variable heavy chain domain (V_H) and the variable light chain domain (V_L). The heterodimers of heavy and light chain domains that occur in whole IgG, for example, are connected by a disulfide bond. Recombinant Fvs in which V_H and V_L are connected by a peptide linker are typically stable. These are single chain Fvs which have been found to retain specificity and affinity and have been shown to be useful for imaging tumors and to make recombinant immunotoxins for tumor therapy. However, researchers have bound that some of the single chain Fvs have a reduced affinity for antigen and the peptide linker can interfere with binding. Improved Fv's have been also been made which comprise stabilizing disulfide bonds between the V_H and V_L regions, as described in U.S. Patent No. 6,147,203, incorporated fully herein by reference. Any of these minimal antibodies may be utilized in the present invention, and those which are humanized to avoid HAMA reactions are preferred for use in embodiments of the invention.

In addition, derivatized immunoglobulins with added chemical linkers, detectable moieties, such as fluorescent dyes, enzymes, substrates, chemiluminescent moieties and the like, or specific binding moieties, such as streptavidin, avidin, or biotin, and the like may be utilized in the methods and compositions of the present invention. For convenience, the term "antibody" or "antibody moiety" will be used throughout to generally refer to molecules which specifically bind to an epitope of the brain tumor protein targets, although the term will encompass all immunoglobulins, derivatives, fragments, recombinant or engineered immunoglobulins, and modified immunoglobulins, as described above.

Candidate antibodies can be tested for activity by any suitable standard means. As a first screen, the antibodies may be tested for binding against the immunogen. As a second screen, antibodies may be screened for cross-reactivity between alleles and between TIM family members, and tested for activity in inhibition of TIM function. For these screens, the candidate antibody may be labeled for detection. Antibodies that alter the biological activity of a TIM protein may be assayed in functional formats.

DIAGNOSIS

Diagnosis of asthma or atopy associated with Tim polymorphisms is performed by protein, DNA or RNA sequence and/or hybridization analysis of any convenient sample from a patient, *e.g.* biopsy material, blood sample, scrapings from cheek, *etc.* A nucleic acid sample from a patient having asthma that may be associated with *TIM,* is analyzed for the presence of a predisposing polymorphism in *TIM.* A typical patient genotype would have at least one predisposing mutation on at least one chromosome. The presence of a polymorphic *TIM* sequence that affects the activity or

expression of the gene product, and confers an increased susceptibility to asthma is considered a predisposing polymorphism. Individuals are screened by analyzing their DNA or mRNA for the presence of a predisposing polymorphism, as compared to an asthma neutral sequence. Specific sequences of interest include any polymorphism that leads to clinical bronchial hyperreactivity or is otherwise associated with asthma, including, but not limited to, insertions, substitutions and deletions in the coding region sequence, intron sequences that affect splicing, or promoter or enhancer sequences that affect the activity and expression of the protein. Examples of specific *TIM* polymorphisms are provided in the Examples.

Screening may also be based on the functional or antigenic characteristics of the protein. Immunoassays designed to detect predisposing polymorphisms in TIM proteins may be used in screening. Where many diverse mutations lead to a particular disease phenotype, functional protein assays have proven to be effective screening tools.

Biochemical studies may be performed to determine whether a candidate sequence polymorphism in the *TIM* coding region or control regions is associated with disease. For example, a change in the promoter or enhancer sequence that affects expression of *TIM* may result in predisposition to asthma. Expression levels of a candidate variant allele are compared to expression levels of the normal allele by various methods known in the art. Methods for determining promoter or enhancer strength include quantitation of the expressed natural protein; insertion of the variant control element into a vector with a reporter gene such as β-galactosidase, luciferase, chloramphenicol acetyltransferase, *etc.* that provides for convenient quantitation; and the like. The activity of the encoded TIM protein may be determined by comparison with the wild-type protein.

A number of methods are available for analyzing nucleic acids for the presence of a specific sequence. Where large amounts of DNA are available, genomic DNA is used directly. Alternatively, the region of interest is cloned into a suitable vector and grown in sufficient quantity for analysis. Cells that express *TIM* genes, such as trachea cells, may be used as a source of mRNA, which may be assayed directly or reverse transcribed into cDNA for analysis. The nucleic acid may be amplified by conventional techniques, such as the polymerase chain reaction (PCR), to provide sufficient amounts for analysis. The use of the polymerase chain reaction is described in Saiki, *et al.* (1985) Science **239**:487, and a review of current techniques may be found in Sambrook, *et al.* Molecular Cloning: A Laboratory Manual, CSH Press 1989, pp.14.2-14.33. Amplification may also be used to determine whether a polymorphism is present, by using a primer that is specific for the polymorphism. Alternatively, various methods are known in the art that utilize oligonucleotide ligation as a means of detecting polymorphisms, for examples see Riley *et al.* (1990) N.A.R. **18**:2887-2890; and Delahunty *et al.* (1996) Am. J. Hum. Genet.

58:1239-1246.

A detectable label may be included in an amplification reaction. Suitable labels include fluorochromes, *e.g.* fluorescein isothiocyanate (FITC), rhodamine, Texas Red, phycoerythrin, allophycocyanin, 6-carboxyfluorescein (6-FAM), 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE), 6-carboxy-X-rhodamine (ROX), 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 5-carboxyfluorescein (5-FAM) or N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), radioactive labels, *e.g.* ^{32}P, ^{35}S, ^{3}H; *etc.* The label may be a two stage system, where the amplified DNA is conjugated to biotin, haptens, *etc.* having a high affinity binding partner, *e.g.* avidin, specific antibodies, *etc.*, where the binding partner is conjugated to a detectable label. The label may be conjugated to one or both of the primers. Alternatively, the pool of nucleotides used in the amplification is labeled, so as to incorporate the label into the amplification product.

The sample nucleic acid, *e.g.* amplified or cloned fragment, is analyzed by one of a number of methods known in the art. The nucleic acid may be sequenced by dideoxy or other methods, and the sequence of bases compared to a neutral *TIM* sequence. Hybridization with the variant sequence may also be used to determine its presence, by Southern blots, dot blots, *etc.* The hybridization pattern of a control and variant sequence to an array of oligonucleotide probes immobilised on a solid support, as described in US 5,445,934, or in WO95/35505, may also be used as a means of detecting the presence of variant sequences. Single strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), mismatch cleavage detection, and heteroduplex analysis in gel matrices are used to detect conformational changes created by DNA sequence variation as alterations in electrophoretic mobility. Alternatively, where a polymorphism creates or destroys a recognition site for a restriction endonuclease (restriction fragment length polymorphism, RFLP), the sample is digested with that endonuclease, and the products size fractionated to determine whether the fragment was digested. Fractionation is performed by gel or capillary electrophoresis, particularly acrylamide or agarose gels.

The hybridization pattern of a control and variant sequence to an array of oligonucleotide probes immobilised on a solid support, as described in US 5,445,934, or in WO95/35505, may be used as a means of detecting the presence of variant sequences. In one embodiment of the invention, an array of oligonucleotides are provided, where discrete positions on the array are complementary to at least a portion of mRNA or genomic DNA of the *TIM* locus. Such an array may comprise a series of oligonucleotides, each of which can specifically hybridize to a nucleic acid, *e.g.* mRNA, cDNA, genomic DNA, *etc.* from the *TIM* locus.

Antibodies specific for TIM polymorphisms may be used in screening

immunoassays. A reduction or increase in neutral TIM and/or presence of asthma associated polymorphisms is indicative that asthma is TIM-associated. A sample is taken from a patient suspected of having TIM-associated asthma. Samples, as used herein, include biological fluids such as tracheal lavage, blood, cerebrospinal fluid, tears, saliva, lymph, dialysis fluid and the like; organ or tissue culture derived fluids; and fluids extracted from physiological tissues. Also included in the term are derivatives and fractions of such fluids. Biopsy samples are of particular interest, *e.g.* trachea scrapings, *etc.* The number of cells in a sample will generally be at least about 10^3, usually at least 10^4 more usually at least about 10^5. The cells may be dissociated, in the case of solid tissues, or tissue sections may be analyzed. Alternatively a lysate of the cells may be prepared.

Diagnosis may be performed by a number of methods. The different methods all determine the absence or presence or altered amounts of normal or abnormal TIM in patient cells suspected of having a predisposing polymorphism in TIM. For example, detection may utilize staining of cells or histological sections, performed in accordance with conventional methods. The antibodies of interest are added to the cell sample, and incubated for a period of time sufficient to allow binding to the epitope, usually at least about 10 minutes. The antibody may be labeled with radioisotopes, enzymes, fluorescers, chemiluminescers, or other labels for direct detection. Alternatively, a second stage antibody or reagent is used to amplify the signal. Such reagents are well known in the art. For example, the primary antibody may be conjugated to biotin, with horseradish peroxidase-conjugated avidin added as a second stage reagent. Final detection uses a substrate that undergoes a color change in the presence of the peroxidase. The absence or presence of antibody binding may be determined by various methods, including flow cytometry of dissociated cells, microscopy, radiography, scintillation counting, *etc.*

An alternative method for diagnosis depends on the *in vitro* detection of binding between antibodies and TIM in a lysate. Measuring the concentration of TIM binding in a sample or fraction thereof may be accomplished by a variety of specific assays. A conventional sandwich type assay may be used. For example, a sandwich assay may first attach TIM-specific antibodies to an insoluble surface or support. The particular manner of binding is not crucial so long as it is compatible with the reagents and overall methods of the invention. They may be bound to the plates covalently or non-covalently, preferably non-covalently.

Other immunoassays are known in the art and may find use as diagnostics. Ouchterlony plates provide a simple determination of antibody binding. Western blots may be performed on protein gels or protein spots on filters, using a detection system specific for TIM as desired, conveniently using a labeling method as described for the sandwich assay.

The *TIM* genes are useful for analysis of TIM expression, *e.g.* in determining developmental and tissue specific patterns of expression, and for modulating expression *in vitro* and *in vivo.* Vectors useful for introduction of the gene include plasmids and viral vectors. Of particular interest are retroviral-based vectors, *e.g.* Moloney murine leukemia virus and modified human immunodeficiency virus; adenovirus vectors, *etc.* that are maintained transiently or stably in mammalian cells. A wide variety of vectors can be employed for transfection and/or integration of the gene into the genome of the cells. Alternatively, micro-injection may be employed, fusion, or the like for introduction of genes into a suitable host cell. See, for example, Dhawan *et al.* (1991) Science **254**:1509-1512 and Smith *et al.* (1990) Molecular and Cellular Biology 3268-3271.

The expression vector will have a transcriptional initiation region oriented to produce functional mRNA. The native transcriptional initiation region or an exogenous transcriptional initiation region may be employed. The promoter may be introduced by recombinant methods *in vitro*, or as the result of homologous integration of the sequence into a chromosome. Many strong promoters are known in the art, including the β-actin promoter, SV40 early and late promoters, human cytomegalovirus promoter, retroviral LTRs, methallothionein responsive element (MRE), tetracycline-inducible promoter constructs, *etc.*

Expression vectors generally have convenient restriction sites located near the promoter sequence to provide for the insertion of nucleic acid sequences. Transcription cassettes may be prepared comprising a transcription initiation region, the target gene or fragment thereof, and a transcriptional termination region. The transcription cassettes may be introduced into a variety of vectors, *e.g.* plasmid; retrovirus, *e.g.* lentivirus; adenovirus; and the like, where the vectors are able to transiently or stably be maintained in the cells, usually for a period of at least about one day, more usually for a period of at least about several days to several weeks.

Antisense molecules are used to down-regulate expression of *TIM* in cells. The anti-sense reagent may be antisense oligonucleotides (ODN), particularly synthetic ODN having chemical modifications from native nucleic acids, or nucleic acid constructs that express such anti-sense molecules as RNA. The antisense sequence is complementary to the mRNA of the targeted gene, and inhibits expression of the targeted gene products. Antisense molecules inhibit gene expression through various mechanisms, *e.g.* by reducing the amount of mRNA available for translation, through activation of RNAse H, or steric hindrance. One or a combination of antisense molecules may be administered, where a combination may comprise multiple different sequences.

Antisense molecules may be produced by expression of all or a part of the

target gene sequence in an appropriate vector, where the transcriptional initiation is oriented such that an antisense strand is produced as an RNA molecule. Alternatively, the antisense molecule is a synthetic oligonucleotide. Antisense oligonucleotides will generally be at least about 7, usually at least about 12, more usually at least about 20 nucleotides in length, and not more than about 500, usually not more than about 50, more usually not more than about 35 nucleotides in length, where the length is governed by efficiency of inhibition, specificity, including absence of cross-reactivity, and the like. It has been found that short oligonucleotides, of from 7 to 8 bases in length, can be strong and selective inhibitors of gene expression (see Wagner *et al.* (1996) Nature Biotechnology **14**:840-844).

TRANSGENIC ANIMALS

The subject nucleic acids can be used to generate genetically modified non-human animals or site specific gene modifications in cell lines. The term "transgenic" is intended to encompass genetically modified animals having a deletion or other knock-out of *TIM* gene activity, having an exogenous *TIM* gene that is stably transmitted in the host cells, or having an exogenous *TIM* promoter operably linked to a reporter gene. Transgenic animals may be made through homologous recombination, where the *TIM* locus is altered. Alternatively, a nucleic acid construct is randomly integrated into the genome. Vectors for stable integration include plasmids, retroviruses and other animal viruses, YACs, and the like. Of interest are transgenic mammals, *e.g.* cows, pigs, goats, horses, *etc.*, and particularly rodents, *e.g.* rats, mice, *etc.*

A "knock-out" animal is genetically manipulated to substantially reduce, or eliminate endogenous *TIM* function. Different approaches may be used to achieve the "knock-out". A chromosomal deletion of all or part of the native *TIM* homolog may be induced. Deletions of the non-coding regions, particularly the promoter region, 3' regulatory sequences, enhancers, or deletions of gene that activate expression of TIM genes. A functional knock-out may also be achieved by the introduction of an anti-sense construct that blocks expression of the native *TIM* genes (for example, see Li and Cohen (1996) Cell 85:319-329).

Transgenic animals may be made having exogenous *TIM* genes. The exogenous gene is usually either from a different species than the animal host, or is otherwise altered in its coding or non-coding sequence. The introduced gene may be a wild-type gene, naturally occurring polymorphism, or a genetically manipulated sequence, for example those previously described with deletions, substitutions or insertions in the coding or non-coding regions. The introduced sequence may encode an TIM polypeptide, or may utilize the *TIM* promoter operably linked to a reporter gene. Where the introduced gene is a coding sequence, it usually operably linked to a promoter, which may be

constitutive or inducible, and other regulatory sequences required for expression in the host animal.

Specific constructs of interest, but are not limited to, include anti-sense *TIM*, which will block TIM expression, expression of dominant negative TIM mutations, and over-expression of a TIM gene. A detectable marker, such as *lac Z* may be introduced into the *TIM* locus, where upregulation of *TIM* expression will result in an easily detected change in phenotype.

The modified cells or animals are useful in the study of *TIM* function and regulation. Animals may be used in functional studies, drug screening, *etc.*, *e.g.* to determine the effect of a candidate drug on asthma. A series of small deletions and/or substitutions may be made in the *TIM* gene to determine the role of different exons in DNA binding, transcriptional regulation, *etc.* By providing expression of TIM protein in cells in which it is otherwise not normally produced, one can induce changes in cell behavior. These animals are also useful for exploring models of inheritance of asthma, *e.g.* dominant *v.* recessive; relative effects of different alleles and synergistic effects between *TIM* and other asthma genes elsewhere in the genome.

DNA constructs for homologous recombination will comprise at least a portion of the *TIM* gene with the desired genetic modification, and will include regions of homology to the target locus. DNA constructs for random integration need not include regions of homology to mediate recombination. Conveniently, markers for positive and negative selection are included. Methods for generating cells having targeted gene modifications through homologous recombination are known in the art. For various techniques for transfecting mammalian cells, see Keown *et al.* (1990) Methods in Enzymology **185**:527-537.

Drug screening may be performed in combination with the subject animal models. Many mammalian genes have homologs in yeast and lower animals. The study of such homologs' physiological role and interactions with other proteins can facilitate understanding of biological function. In addition to model systems based on genetic complementation, yeast has been shown to be a powerful tool for studying protein-protein interactions through the two hybrid system described in Chien *et al.* (1991) P.N.A.S. **88**:9578-9582. Two-hybrid system analysis is of particular interest for exploring transcriptional activation by *TIM* proteins.

COMPOUND SCREENING

One can identify ligands or substrates that bind to, modulate or mimic the action of TIM. Areas of investigation are the development of treatments for immune disorders, asthma, cancer, ischemia-reperfusion injury, and other diseases that are

associated with cellular responses to stress. Drug screening identifies agents that provide an inhibition, replacement, or enhancement for TIM function in affected cells. For example, agents that reverse or inhibit TIM function may reduce bronchial reactivity in asthma by reducing levels of Th2 cytokines, and TIM inhibitors may enhance tumor sensitivity to cancer therapy, by potentiating the effects of radiation and chemotherapeutic treatments that induce apoptosis. Of particular interest are screening assays for agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including labeled *in vitro* protein-protein binding assays, protein-DNA binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like. The purified protein may also be used for determination of three-dimensional crystal structure, which can be used for modeling intermolecular interactions, transcriptional regulation, *etc.*

The term "agent" as used herein describes any molecule, *e.g.* protein or pharmaceutical, with the capability of altering or mimicking the physiological function of TIM, such as a signal tyrosine kinase inhibitor, or a peptide inhibitor of an integrin binding site. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, *i.e.* at zero concentration or below the level of detection.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including, but not limited to: peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical

modifications, such as acylation, alkylation, esterification, amidification, *etc.* to produce structural analogs.

Where the screening assay is a binding assay, one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, specific binding molecules, particles, *e.g.* magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule that provides for detection, in accordance with known procedures.

A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc that are used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, *etc.* may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening. Typically between 0.1 and 1 hours will be sufficient.

Other assays of interest detect agents that mimic TIM function. For example, candidate agents are added to a cell that lacks functional TIM, and screened for the ability to reproduce TIM in a functional assay.

THERAPEUTIC METHODS

Agents that modulate activity of TIM genes or proteins provide a point of therapeutic or prophylactic intervention, particularly agents that inhibit or upregulate activity of the polypeptide, or expression of the gene. Numerous agents are useful in modulating this activity, including agents that directly modulate expression, *e.g.* expression vectors, antisense specific for the targeted polypeptide; and agents that act on the protein, *e.g.* specific antibodies and analogs thereof, small organic molecules that block catalytic activity, *etc.*

Methods can be designed to selectively deliver nucleic acids to certain cells. Examples of such cells include T cells, etc. Certain treatment methods are designed to selectively express an expression vector to cells of interest. One technique for achieving selective expression in nerve cells is to operably link the coding sequence to a promoter that is primarily active in immune system cells, e.g. IL-2 promoter, T cell antigen receptor promoter, and the like. Alternatively, or in addition, the nucleic acid can be administered with an agent that targets the nucleic acid to the cells of interest. For instance, the nucleic

acid can be administered with an antibody that specifically binds to a cell-surface antigen. When liposomes are utilized, substrates that bind to a cell-surface membrane protein associated with endocytosis can be attached to the liposome to target the liposome to nerve cells and to facilitate uptake.

Antisense molecules can be used to down-regulate expression in cells. The antisense reagent may be antisense oligonucleotides (ODN), particularly synthetic ODN having chemical modifications from native nucleic acids, or nucleic acid constructs that express such antisense molecules as RNA. The antisense sequence is complementary to the mRNA of the targeted gene, and inhibits expression of the targeted gene products. Antisense molecules inhibit gene expression through various mechanisms, e.g. by reducing the amount of mRNA available for translation, through activation of RNAse H, or steric hindrance. One or a combination of antisense molecules may be administered, where a combination may comprise multiple different sequences.

Antisense molecules may be produced by expression of all or a part of the target gene sequence in an appropriate vector, where the transcriptional initiation is oriented such that an antisense strand is produced as an RNA molecule. Alternatively, the antisense molecule is a synthetic oligonucleotide. Antisense oligonucleotides will generally be at least about 7, usually at least about 12, more usually at least about 20 nucleotides in length, and not more than about 500, usually not more than about 50, more usually not more than about 35 nucleotides in length, where the length is governed by efficiency of inhibition, specificity, including absence of cross-reactivity, and the like. It has been found that short oligonucleotides, of from 7 to 8 bases in length, can be strong and selective inhibitors of gene expression (see Wagner *et al.* (1996) Nature Biotechnology **14**:840-844).

A specific region or regions of the endogenous sense strand mRNA sequence is chosen to be complemented by the antisense sequence. Selection of a specific sequence for the oligonucleotide may use an empirical method, where several candidate sequences are assayed for inhibition of expression of the target gene *in vitro* or in an animal model. A combination of sequences may also be used, where several regions of the mRNA sequence are selected for antisense complementation.

Antisense oligonucleotides may be chemically synthesized by methods known in the art (see Wagner *et al.* (1993) supra. and Milligan *et al.*, supra.) Preferred oligonucleotides are chemically modified from the native phosphodiester structure, in order to increase their intracellular stability and binding affinity. A number of such modifications have been described in the literature, which alter the chemistry of the backbone, sugars or heterocyclic bases.

Among useful changes in the backbone chemistry are phosphorothioates; phosphorodithioates, where both of the non-bridging oxygens are substituted with sulfur;

phosphoroamidites; alkyl phosphotriesters and boranophosphates. Achiral phosphate derivatives include 3'-O'-5'-S-phosphorothioate, 3'-S-5'-O-phosphorothioate, 3'-CH2-5'-O-phosphonate and 3'-NH-5'-O-phosphoroamidate. Peptide nucleic acids replace the entire ribose phosphodiester backbone with a peptide linkage. Sugar modifications are also used to enhance stability and affinity. The alpha.-anomer of deoxyribose may be used, where the base is inverted with respect to the natural .beta.-anomer. The 2'-OH of the ribose sugar may be altered to form 2'-O-methyl or 2'-O-allyl sugars, which provides resistance to degradation without comprising affinity. Modification of the heterocyclic bases must maintain proper base pairing. Some useful substitutions include deoxyuridine for deoxythymidine; 5-methyl-2'-deoxycytidine and 5-bromo-2'-deoxycytidine for deoxycytidine. 5-propynyl-2'-deoxyuridine and 5-propynyl-2'-deoxycytidine have been shown to increase affinity and biological activity when substituted for deoxythymidine and deoxycytidine, respectively.

The compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host for treatment of asthma attributable to a defect in TIM function. The compounds may also be used to enhance TIM function. The therapeutic agents may be administered in a variety of ways, orally, topically, parenterally *e.g.* subcutaneously, intraperitoneally, by viral infection, intravascularly, *etc.* Inhaled treatments are of particular interest. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%.

The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents.

It is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, animal species or genera, and reagents described, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

As used herein the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a

cell" includes a plurality of such cells and reference to "the array" includes reference to one or more arrays and equivalents thereof known to those skilled in the art, and so forth. All technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs unless clearly indicated otherwise.

All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing, for example, the cell lines, constructs, and methodologies that are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the subject invention, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to ensure accuracy with respect to the numbers used (*e.g.* amounts, temperature, concentrations, *etc.*) but some experimental errors and deviations should be allowed for. Unless otherwise indicated, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees centigrade; and pressure is at or near atmospheric.

EXPERIMENTAL

To analyze the human 5q23-35 region for asthma susceptibility genes, we utilized a mouse model, which offers several potential advantages. Environmental variation can be controlled, multiple phenotypes can be tested simultaneously, and inbred strains can be sensitized with allergen to develop airway hyperreactivity (AHR), a cardinal feature of human asthma. We utilized congenic inbred mouse strains that differed only by a small chromosomal region homologous to human chromosome 5q, thereby allowing this region to be studied in the absence of genetic variation outside the region. Positional cloning revealed a novel gene family encoding T cell membrane proteins (*Tim*), TIM-1, TIM-2, TIM-3, TIM-4, TIM-5, TIM-6, and TIM-7, in which major sequence variants of TIM-1, TIM-3, and TIM-4, cosegregate completely with *Tapr.*

IL-4 production and airway hyperreactivity are reduced in HBA mice. We examined congenic mice produced on a BALB/c genomic background with discrete genomic intervals inherited from individual DBA/2 chromosomes. BALB/c mice develop Th2 biased, atopy-resembling immune responses with enhanced AHR, while DBA/2 mice

develop reduced IL-4 responses that protect against the development of AHR. By screening several of these congenic strains for reduced Th2 responsiveness, we identified one congenic line, C.D2 Es-Hba (HBA), which contained a segment of chromosome 11 inherited from DBA/2 mice, homologous to human 5q23-35. Fig. 1a shows that lymph node cells from immunized control BALB/c mice, as expected, produced high levels of IL-4, confirming the proclivity of BALB/c mice to develop Th2-biased immune responses. In contrast, lymph node cells from HBA mice produced significantly lower levels of IL-4, similar to that observed in DBA/2 mice. In addition, HBA mice produced significantly less IL-13 and IL-10, and somewhat lower levels of IL-5 compared to BALB/c mice, whereas production of IFN-γ was increased, as shown in Fig. 1b. These results indicated that the DBA/2-derived region of HBA chromosome 11, which has large regions of conserved synteny with human 5q23-35, contains a gene that reduces antigen-specific IL-4, IL-13, and IL-10 production, enhances IFN-γ production, and converts the BALB/c cytokine phenotype into a DBA/2 cytokine phenotype.

The HBA mice were examined for the capacity to develop antigen-induced airway hyperreactivity (AHR), which is associated with Th2-biased immune responses. Upon sensitization and challenge with allergen, control BALB/c mice developed high AHR, whereas similarly immunized HBA congenic mice, like DBA/2 mice, expressed normal airway reactivity in response to methacholine (Fig. 1c). Collectively, these results strongly suggested that genetic variation in a single locus on chromosome 11 regulated both Th2 cytokine production and AHR; therefore, we tentatively refer to the relevant genetic determinant(s) in HBA mice as a single locus, *T cell and Airway Phenotype Regulator (Tapr).*

We also examined (BALB/c x HBA) F1 mice, which like the BALB/c mice, produced high levels of IL-4, IL-13, and IL-10 (Fig. 1a and 1b) and developed elevated antigen-induced AHR (Fig. 1c). These results indicate that a DBA/2 allele on chromosome 11, in isolation of other genes that regulate IL-4 synthesis, reduced IL-4 production and AHR in a recessive manner. In contrast, (BALB/c x DBA/2) F1 mice produced low levels of IL-4 and had normal airway responsiveness on immunization (Fig. 1), indicating that loci from other regions of the DBA genome also modulated IL-4 production and antigen-induced AHR, and that the DBA/2 alleles, in aggregate, functioned in a dominant manner to limit IL-4 production and AHR. These results underscore the multigenic, complex nature of atopic traits and demonstrate the potential advantages of using a congenic strain to isolate and characterize a single locus without interference from multiple epistatic genes that also influence the asthmatic phenotype.

Genetic Mapping of Tapr, the locus which controls AHR and IL-4 responsiveness. Previously, the congenic region in the HBA mice was delineated with 36

genome-wide markers, including two chromosome 11 markers, hemoglobin-α2 (*hba-α2*) and esterase-3 (*es-3*) loci. The HBA genome, outside of chromosome 11, was inherited from BALB/c. A more precise analysis with 25 simple sequence length polymorphism (SSLP) markers known to be polymorphic between DBA/2 and BALB/c mice showed that HBA mice inherited two segments of chromosome 11 from DBA/2 (Fig. 2, left column). The proximal region contained a 20 cM segment with homology to chromosome 5q23-35, which afforded the possibility that a genetic locus implicated in human asthma linkage studies could be identified in a mouse model of asthma.

To map at higher resolution the T_H2-AHR controlling locus, *Tapr*, (BALB/c x HBA) Fl mice were backcrossed to HBA mice to produce N2 animals. With this backcross approach, the set of alleles contributed by the HBA parent is pre-determined, and the set of alleles contributed by the F1 parent can be assessed by genotyping. Thus, recombination events that produce informative haplotypes within the congenic region can be detected in the N2 mice and used to assess the linkage of *Tapr* to loci in the congenic interval. Because of the recessive nature of *Tapr*, we tested N2 mice from these backcrosses to identify the minimum homozygous region of HBA-derived genes sufficient to confer the HBA *Tapr* phenotype. More than 2,000 N2 animals were generated and genotyped. Using SSLP markers, we selected those N2 mice with informative recombination events, and the N2 mice were phenotyped for the capacity to produce IL-4 in response to immunization with keyhole limpet hemocyanin (KLH). In this primary analysis, we determined that the relevant locus resided within the proximal congenic region, between *D11Mit135* and *D11Mit260*. In order to map *Tapr* at higher resolution, 22 additional markers were identified and utilized to provide 0.1-1 cM resolution in the area of interest.

To accurately compare the results of IL-4 cytokine analyses performed over several months time, an IL-4 index for each experiment was generated for each N2 mouse, $\left(\frac{B-x}{B-H}\right)$, where B=IL-4 production by cells from BALB/c mice, H=IL-4 production by cells from HBA mice, and *x*=IL-4 production by cells from the N2 mouse being assessed. High concentrations of IL-4 (BALB/c-like) are represented by index values near 0, and low concentrations of IL-4 (HBA-like) are represented by index values near 1.0. The "B and "H" values were established with 3-5 control mice for each group of 3-6 N2 mice carrying informative recombinations that we tested. The index values fall within a bimodal distribution (Fig. 3a), in which the phenotype index associated with N2 mice that had nonrecombinant HBA genotypes was significantly higher ($P<0.0001$, in a paired Student's t-test) than the phenotype index associated with N2 mice that had nonrecombinant (BALB/c x HBA)F1 genotypes.

For the mice with unique genotypes, we used several methods to ensure the

adequacy of single measurements of cytokine production and AHR, since this is critical in linkage analysis. First, at the same time that we tested each of the N2 mice carrying recombinations of interest, we also tested "non-recombinant" siblings of each "recombinant N2" that were strictly HBA or F1 (BALB x HBA) in genotype. Furthermore, we bred additional N3 mice by crossing some of the N2 mice carrying recombinations of interest back to HBA mice, in order to have more individual mice with that particular N2 genotype. All values were the average of the values for the individual mice tested with a given genotype. In this way, we are confident of the measures of cytokine production and AHR, and that we have overcome assay variations due to variables inherent in biological systems.

Because the IL-4 values associated with the N2 mice that inherited recombinant haplotypes segregated in a bimodal distribution (Fig. 3a), were able to demonstrate that the genetic locus that controls high IL-4 responses is located between markers *D11Mit271* and *D11Mit22* (Fig. 3b). Moreover, high levels of IL-4 production were observed in all mice with a BALB/c allele present at *Kim1sscp,* and low levels of IL-4 production were observed in all mice with homozygous HBA genotypes at *Kim1sscp.* Thus, *Tapr* was nonrecombinant with *Kim1sscp,* an intronic marker within a mouse homologue of *Rattus norvegicus* Kidney Injury Molecule (*Kim-1*). In contrast, *Tapr* segregated from all other markers with at least one recombination. The fact that *Tapr* and *Kim1sscp* segregated together, indicated that the *Tapr* locus is located very close to or is indistinguishable from *Kim1sscp.* Based on the frequency recombinant haplotypes between *D11Mit271* and *D11Mit22,* we calculate a recombination frequency, 0.0039, which indicates that that the *Tapr* locus maps to a small, 0.3-0.5 cM, region. We also calculated a recombination frequency of 0.08 between *Tapr* and IL-4. Therefore, *Tapr* is located 5-10 cM away from the IL-4 cytokine cluster but is within the a region of the mouse genome that has highly conserved synteny with the 5q23-35 region that has been linked to human atopy and asthma.

Using an analogous approach, we examined the segregation of allergen-induced AHR phenotypes in mice with informative recombinant haplotypes. With indexed AHR values, N2 mice clearly exhibit parental phenotypes, which produced a bimodal distribution in a histogram of AHR index values in a group of sensitized N2 mice (Fig. 3c). By analyzing the segregation of AHR phenotypes associated with more than 1,000 N2 mice, we demonstrated that the genetic locus which controls AHR responses is also located between markers *D11Mit271* and *D11Mit22* (Fig. 3d) and that the AHR phenotype was nonrecombinant with *Kim1sscp.* Thus, we demonstrate that both IL-4 responsiveness and AHR cosegregate with the *Tapr* locus, which suggests that the same locus regulates both IL-4 expression and AHR (Fig. 3).

These findings further demonstrate that the *Tapr* locus is more than 5 cM centromeric to the IL-4 cytokine cluster and the cytokine genes in the cluster previously thought to be 'candidate' atopy or asthma susceptibility genes. Our mapping results also establish that *Tapr* is genetically separable from both the IL-12p40 gene and the region of mouse chromosome 11 that includes the T_H1-IL12 regulatory locus, *Tpm*.

Mouse and human homologues anchor Tapr to human 5q33. In order to construct a composite map around the *Tapr* locus, we integrated available information from the Mouse Genome Database (MGD) linkage, backcross, and radiation hybrid maps and identified a region of conserved synteny in maps of the human genome. Current radiation hybrid maps place the markers that are near *D11Mit271* and *D11Mit22,* including several expressed sequence tags (ESTs) that have extensive homology to known genes or unigene clusters, onto a physical map of the mouse genome. We further examined these markers and their associated ESTs for previously unidentified similarity to known gene clusters. We assembled these markers onto a scaffold for comparison to the human genome. Using this approach, we found significant similarity between particular radiation hybrid markers and the following human genes: *KIAA0171, Adam-19, Sox-30, Pir-121, Crsp9* (*Crsp33*), and *hHAVcr-1* (*hHAVcr-1*). Fig. 4 demonstrates that once we anchored these genes to a physical map of the mouse genome between our flanking markers, we were able to locate those genes in the Human Genome Browser.

The high degree of conservation between the mouse and human genomes in this region indicates linkage of the *Tapr* locus to human 5q33.2. As shown Fig. 4, we identified all known genes and ESTs in this region of the human map. Genes of particular interest near human *hHAV-cr* and the mouse homologue of *Kim-1*, include IL-2 inducible T cell kinase (*Itk*) and a coregulator of the SP-1 transcription factor (*Crsp9*), both known to be involved in T cell differentiation. We sequenced coding regions from these candidate genes and found no polymorphisms in either ITK or CRSP-9.

Localization of a Family of Novel T cell Surface Proteins to the Tapr Region. Because the mouse homolog of rat *Kim1* is located within the 0.4 cM region and is tightly linked with *Tapr,* we examined publicly available databases and found clusters of ESTs with some sequence similarity that provided only partial coverage and contained large segments of variation. The closest human homolog of *Kim-1* is the human hepatitis A virus cellular receptor, *hHAV-cr*, and tBLAST searches of the human genome suggested that two additional homologs of *Kim-1*, perhaps members of a gene family, also are located on human chromosome 5 and mouse chromosome 11.

Using cDNA from conA-stimulated splenocytes, we identified and cloned two

mouse orthologues of *Kim1,* which we term *Tim1* and *Tim2,* that map to the *Tapr* region, as shown in Fig. 5A. TIM-3 is a third, more distantly related, orthologue of KIM-1.

All three members of this gene family are expressed by stimulated T cells, and all three forms map to the *Tapr* region of mouse chromosome 11/ human chromosome 5 where they encode cell surface glycoproteins with common structural motifs, including an immunoglobulin (Ig) domain, mucin domain, and intracellular tail with phosphorylation sites. Because the cellular functions of these proteins is unknown, we refer to the genes as members of a *T cell, Immunoglobulin domain, Mucin domain (Tim)* gene family. Mouse *Tim1* is the mouse homologue of rat *Kim1* and the *HAVcr-1* identified in African green monkeys and humans. *Tim2* is a previously unknown gene that had not been identified in any organism prior to this study.

The mouse *Tim1* gene encodes a 305 amino acid membrane protein, that has 78% overall identity with rat KIM-1 and 35% identity with human HAVcr-1. A gapped multiple sequence alignment with mouse TIM-1, rat KIM-1, human HAVcr-1 and African green monkey HAVcr-1, shown in Fig. 5B, demonstrates the degree of homology between theTIM-1/KIM-1/HAVcr-1 proteins in these species. The cytoplasmic region of TIM-1 contains two tyrosine residues and includes a highly conserved tyrosine kinase phosphorylation motif, RAEDNIY, which is integral to the predicted Itk and EGFR kinase site of TIM-1, SRAEDNIYIVEDRP. The mucin domain of TIM-1 has multiple sites for O-linked glycosylation, and there two sites for N-linked glycosylation found in the immunoglobulin domain.

TIM-2 , a similar 305 amino acid membrane protein, has 64% identity to mouse TIM-1, 60% identity to rat KIM-1, and 32% identity to hHAVcr-1 (Figure 5A, B). Like TIM-1, TIM-2 has two extracellular N-linked glycosylation sites and a serine, threonine-rich mucin domain with many O-linked glycosylation sites. TIM-2 also has an intracellular tyrosine kinase phosphorylation motif, RTRCEDQVY.

Tim3 encodes a 281 amino acid membrane protein that has a similar, integral membrane glycoprotein structure with multiple extracellular glycosylation sites and an intracellular tyrosine phosphorylation motif. Although the mucin domain is not as prominent in TIM-3 as it is in TIM-1 and TIM-2 (Figure 5A), TIM-3 expressed on T cells likely interacts with a ligand on APCs and alters APC activation. TIM-3 does have four sites for N-linked and five sites for O-linked glycosylation, suggesting that TIM-3, like TIM-1 and TIM-2, is heavily glycosylated and might interact with a ligand present on other cells, such as antigen presenting cells.

Tim4 encodes a 344 amino acid protein in mice, and a 378 amino acid protein in humans. The predicted TIM-4 also shares the general membrane glycoprotein structural motifs of the other TIM proteins, a with an IgV-like domain with highly conserved

cysteine residues, a threonine-rich mucin-like domain, and a short intracellular tail. However, TIM-4 lacks the phosphotyrosine motif present in the other TIM proteins, and therefore may modulate the funtion of the other TIM proteins.

Each of the TIM Ig domain shares an predicted integrin-binding motif that is similar to the SVVYGLR motif found in osteopontin, an transmembrane protein like the TIMs that is implicated in the regulation of cell adhesion, survival, and oncogenesis, as well as in the regulation of helper T cell differentiation. This integrin binding motif demonstrates alpha(9) and alpha(4) specificity.

Comparison of the sequences of the BALB/c and HBA/DBA coding regions for the three *Tim* genes revealed major polymorphisms in TIM-1, TIM-3, and TIM-4, but not TIM-2. In TIM-1, these polymorphisms encode three amino acid differences and a fifteen amino acid deletion in HBA/DBA. Seven predicted amino acid differences were identified in TIM-3 (Fig. 5c). Genomic sequences confirm that these polymorphisms, including the deletion, are true polymorphisms, not splicing variants. By further sequencing genomic segments of TIM-1 and TIM-3 in other mouse strains, we found that C57/BL6, a strain similar to DBA/2 with respect to its tendency to develop reduced T_H2 and AHR responses, also has the HBA/DBA allele of *Tim1* and *Tim3.* The polymorphisms in TIM-1 and TIM-4 are located in the signal and mucin-like domains, while the polymorphisms identified in TIM-3 are clustered in the Ig domain (Fig. 5c). In glycoproteins with Ig and mucin domains, variants in either domain may affect receptor-ligand interactions, as shown for MAdCAM-1. Although the predicted cleavage sites of TIM-1 and TIM-4 are unaltered by the polymorphism in the signal sequence, it is possible that the polymorphism may affect the efficiency of cleavage and/or trafficking of the receptor to the cell surface. These *Tim* sequences and polymorphisms are important for immune responses, and for HAV viral pathogenesis in humans.

Analysis of genomic DNA samples from our N2 backcross (Fig. 3) demonstrated that the TIM-1 and TIM-3 polymorphisms cosegregate completely with *Tapr.* While these observations do not distinguish the extent to which changes in TIM-1, TIM-3, or both, are responsible for changes in AHR and T_H2-mediated inflammation, we suggest that polymorphisms in human TIM-1(hHAVcr-1) and/or TIM-3 underlie the strong association between asthma susceptibility and human chromosome 5q. This idea is supported by the fact that major variants in coding regions of human *Tim1* are evident on examination of human genome and EST databases. Comparison of these human cDNA variants with the previously described variants of monkey HAVcr-1 and the mouse variants identified here demonstrates that there is extensive variation in the predicted protein sequences of TIM-1 (Fig 5b,c). This high degree of variation distinguishes TIM-1 and its family members from

many other candidate genes, such as the cytokines and the cytokine receptors that have been most closely studied as asthma susceptibility candidate genes. In addition, the association between *Tim1* and asthma susceptibility is further supported by reports of significant linkage of mite-sensitive childhood asthma to D5S820 (mean LOD score = 4.8), a marker which is approximately 0.5 megabases from *Tim1* and *Tim3* (Fig. 4.

In addition to the above genetic polymorphisms, there are several expression polymorphisms in the TIM genes that arise due to alternate splicing. Alternate splicing of TIM-1, TIM-2 and TIM-4 mRNA produces several TIM variants, some of which are secreted, soluble forms of the TIM receptors. These splice variants, along with TIM splice variants that have alternate 5' untranslated regions, may contribute to the cell-specific and condition-specific expression patterns of the TIM proteins.

T cells confer the Tapr effect. To better understand the function of the *Tapr* locus we determined whether allelic variation of *Tapr* affected the function of T cells or of antigen presenting cells (APC). For these experiments, we generated ovalbumin (OVA)-specific T cell receptor (TCR) transgenic mice (Tg) with the HBA background (HBA DO11.10), which we compared to TCR-Tg mice with the BALB/c background (BALB/c DO11.10). Purified CD4+ T cells from either of these strains were cocultured with OVA and dendritic cells (DCs) derived from either BALB/c or HBA bone marrow, and the cytokines produced were evaluated. Irradiated spleen cells were not used as APCs for this experiment, because it was found that irradiated spleen cells and other tissues express high levels of the TIM genes.

BALB/c DO11.10 T cells produced higher levels of IL-4 and IL-13 than did HBA DO11.10 T cells, in a manner that was independent of the source of the antigen presenting cells (Fig. 6A). In addition, the source of the CD4 T cells determined the amount of IL-4/IL-13 produced at each antigen concentration, regardless of the source of the APC during either the primary or secondary stimulation. Equivalent levels of IL-12 were detected in culture supernatants for each combination of cell types, further demonstrating that BALB/c and HBA DC function were comparable. Furthermore, BALB DO11.10 and HBA DO11.10 T cells produced equivalent levels of IL-2 and demonstrated comparable levels of proliferation in response to OVA during the secondary cultures, indicating that HBA and BALB/c T cells are similarly activated, although the levels of Th2 cytokines they produce are quite distinct.

We show in Figure 6B that within the first twelve hours of primary culture in our DO11.10/DC system, we find that mRNA for TIM-1 is expressed by both BALB/c and HBA CD4+ T cells. Within four days of primary stimulation , we find significant levels of IL-13 in supernatants of the BALB/c DO11.10 and detect none in the HBA DO11.10

supernatants. This differentiation is detectable in mRNA levels at 36 hours (Fig. 6B). Between twelve and thirty six hours, expression of IL-13 mRNA is reduced in HBA CD4 T cells, while IL-13 expression is maintained in the BALB/c CD4 T cells. Thus, during the primary response to antigen, BALB/c CD4 T cells develop a stronger Th2 response than do HBA CD4 T cells. Our findings demonstrate that *Tapr* regulates helper T cell differentiation during primary antigen specific responses, and we detect TIM-1 expression in CD4 T cells during the earliest stages of these responses.

Following differentiation into mature Th1 and Th2 subsets, helper T cells demonstrate committed TIM expression by RT-PCR, such that Th1 cells express TIM-3, while Th2 cells preferentially express TIM-1. All T cell populations demonstrate weak TIM-4 expression. While the Itk signal through TIM-1 is likely to promote Th2 differentiation, the EGFR signal through the TIM proteins is likely to enhance cell survival in effector and especially memory T cell populations. Since Itk is expressed only in T cell and mast cells, the Itk kinase activity on TIM-1 is restricted to immune cells, particularly those involved in asthma and allergy. However, other protein tyrosine kinases, such as EGFR, are involved in the function of TIM proteins expressed by other tissues, including ishemic epithelial cells, irradiated spleen cells, and tumor cells.

In these studies, we mapped *Tapr*, a locus that regulates the development of Th2 cytokine production and antigen-induced AHR, a cardinal feature of asthma. We localized *Tapr* using an interval specific congenic mouse (HBA) that carried a chromosomal segment homologous to human chromosome 5q, a region of the human genome that has been repeatedly linked to atopy and asthma. This region has also been repeatedly linked to 5q- syndrome associated with myelodysplasia and neoplastic cytogenic abnormalities, Using this congenic mouse strategy that converted a complex trait into a simpler, possibly single gene, trait, we narrowed the interval of *Tapr* to 0.4 cM interval, sequenced several candidate genes in this region, and positionally cloned the TIM gene family.

The TIM gene family has not been previously described. We identified and cloned the full cDNA sequence and discovered significant polymorphisms in the TIM-1 proteins of BALB/c compared to HBA mice. We found that the BALB/c sequences for TIM-1 and TIM-3 are associated with susceptibility to AHR and allergic T cell responses, whereas the HBA sequences are associated with protection against these responses. TIM-3 is preferentially expressed by differentiated T_H1. The association of polymorphic *Tim3* variants with *Tapr* suggests that TIM-3 might regulate T_H1 and T_H2 cell function. However, the variations in *Tim3* might also be attributed to a haplotype tightly linked to *Tim4* or *Tim1*.

We believe that TIM-1 plays a very important role in the regulation of the immune system (particularly with respect to asthma and allergic disease) and in the the

regulation of epithelial and hematopoetic cell survival in response to stress (hypoxia, nutritional deficiency, irradiation, chemotherapy, etc.) for several reasons. First, *Tim1*, like *Tim3*, is expressed in CD4 T cells during primary antigen stimulation, when it is most likely that the *Tapr* effect occurs. T cells play a critical role in the development of AHR and in the pathogenesis of asthma, our results suggest that *Tapr* affects asthma by enhancing early CD4 commitment to Th2 responses by controlling the production of IL-13 and subsequent T cell differentiation. Second, HAV infection in humans during infancy or childhood is inversely associated with the development of asthma and allergy. We suggest that the HAV interaction with TIM-1/HAVcr-1 may alter the T cell cytokine production may able to reverse or prevent the biased Th1/Th2 balance in individuals otherwise prone to atopy and asthma. SLAM, a measles virus receptor, is an example of another T cell surface glycoprotein that regulates the Th1/Th2 balance in a manner that may be altered by viral interaction. Because some viral receptors, such as SLAM for the measles virus or CD4, CCR5, and CXCR4 for HIV, are receptors of the host's own immune system, even when an infection does not succeed, virus-receptor mediated signal transduction can lead to the release of cytokines and the development of disease.

Third, the polymorphisms in TIM-1 are associated with the different types of helper T cell responses that we observe. Therefore, the variants of TIM-1 may themselves contribute to the genetic Th1/Th2 predisposition that occurs in the absence of any known environmental cause of immune deviation. The HAV receptor in primates is known to be highly variable, and we propose that polymorphic alleles of human TIM-1/hHAVcr-1, like those we have identified in mice, may be associated with variations in Th2 bias and asthma susceptibility. Mutations in the genes for cell surface molecules that serve as viral receptors and that alter susceptibility to infection are not uncommon, and therefore significant genetic variation in TIM-1 and other members of the TIM gene family is far more likely to be observed than variation in other genes such as those for cytokines. It is unclear why asthma susceptibility alleles might be prevalent in the human gene pool, but the association of *Tapr* with HAVcr provides an interesting explanation for the persistence of asthma susceptibility alleles. During human evolution certain alleles of the *Tim* gene family may have conferred resistance to atopic diseases and other immune disorders, but selection of those resistance alleles may have been counterbalanced by selection of alternate alleles that confer resistance to viral pathogenesis.

In summary, our studies represent the first successful utilization of a congenic mouse strategy to locate a strong candidate asthma susceptibility gene and overcome the inherent difficulties in the examination of this complex genetic trait. We identified a previously unknown gene family that exists in a region homologous to human

chromosome 5q, and which plays a major role in Th cell development and in asthma susceptibility. While prior studies in humans identified several candidate genes on human chromosome 5q, the *Tim1* gene product identified in our study also provides an explanation for the inverse relationship between HAV infection and reduced asthma susceptibility.

Subpopulations of $CD4^+$ T cells (Th) produce distinct patterns of cytokines, and this has led to the concept of functional heterogeneity among Th cells. Type 1 Th cells (Thl) produce interleukin 2 (IL-2) and/or interferon γ, elicit delayed type hypersensitivity (DTH) responses and activate macrophages. Type 2 Th cells (Th2), on the other hand, produce IL-4, IL-5 and IL-10 and are especially important for IgE production and eosinophilic inflammation, and may suppress cell mediated immunity. Th2 cells are believed to play a pivotal role in the pathogenesis of atopy. Several factors determine whether a T helper cell will differentiate into Th1 versus Th2 during a particular immune response. These include, but are not necessarily restricted to, the cytokine milieu, the strength of the TCR signal and/or antigen density, and the costimulatory pathways. $CD4^+$ T helper cell differentiation into Th1 or Th2 subsets has profound effects on the outcome of atopy, autoimmune diseases, infectious diseases, and graft rejection.

The specific features of immune responses that protect nonatopic individuals from the development of allergic diseases and which could inhibit allergic responses in atopic individuals are poorly understood. Because Th1 cells cross regulate Th2 cells in some systems, allergen-specific Th1 cells have been assumed to regulate allergic disease and asthma. Th1 cells inhibit the development and proliferation of Th2 cells, and IgE production is reciprocally regulated by IL-4 and IFN-γ. This suggests that protection from allergy is due to the development of inhibitory allergen-specific Th1 cells. Allergen-specific T cell clones derived from the peripheral blood of nonallergic individuals have been shown to produce Th1 cytokines. These observations have also supported the hygiene hypothesis of asthma, which suggests that the prevalence of infections, particularly those that induce Th1 responses, are reduced in westernized societies by improved public health measures and the use of vaccines and antibiotics. As a result, Th2 responses and atopy develop more intensely and rapidly in the absence of Th1 mediated responses.

The TIM genes identified herein are also candidate oncogenes. Transfection of cell lines with TIM genes confers resistance to cell death, and the predicted EGFR kinase motif described in TIM-1 provides a probable mechanism by which this cell survival is controlled. Furthermore, TIM-1 demonstrates a significant degree of sequence identity (approximately 20%) and structural similarity (a transmembrane glycoprotein with an IgV domain, mucin/syndecan domain, transmembrane domain, and intracellular domain with similar phosphotyrosine motifs) with TOSO, a protein that protects cells from Fas-mediated apoptosis. Like the TIM genes, TOSO is a likely oncogene, which maps to a

region of the genome with frequent changes in hematologic malignancies and solid tumors.

METHODS

Animals. Congenic lines, including C.D2 Es-HBA were generated by introgressively backcrossing DBA/2N onto a BALB/cAnPt background. BALB/cBy, DBA/2J, and (BALB/c x DBA/2) F1 mice (CByD2F1/J) were obtained from the Jackson Laboratory (Bar Harbor, ME), while BALB/cAn and DBA/2N were obtained from Taconic Labs. (BALB/c x HBA) F1 mice were produced with a cross between BALB/cByJ and HBA. N2 mice were generated by backcrossing (BALB/c x HBA) F1 to HBA. In our analysis of recombinant N2 animals, recombinant mice were tested along with non-recombinant siblings, whenever possible. In order to examine individual N2 genotypes in multiple assays, we preserved selected recombinant haplotypes by backcrossing selected N2 mice to HBA to generate N3 mice, which were genotyped to chose mice carrying the recombinant N2 haplotype. DO11.10 mice, which are transgenic for TCR recognizing OVA peptide 323-339 ($pOVA^{323-339}$) and backcrossed to BALB/c(43), were kindly provided by Dr. Dennis Loh and were bred in our facilities. HBA DO11.10 mice were produced by backcrossing DO11.10 to HBA. DO11.10 mice were selected by FACS analysis for the TCR-Tg and genotyped to select for HBA alleles between *D11Mit135* and *D11Mit168*. The Stanford University Committee on Animal Welfare approved all animal protocols.

Genotyping. Additional markers around the *Tapr* locus were identified by testing all available "D11Mit-" markers present between *D11Mit140* and *D11Mit269* and all radiation hybrid markers near *D11Mit271* and *D11Mit22* for any polymorphisms between DBA/2 and BALB/c. MIT MapPair primers were obtained from Research Genetics (Huntsville, AL), and all other primers were synthesized in the Protein and Nucleic Acid Facility (Stanford, CA). PCR was performed as previously described, and SSLP polymorphisms were resolved with 4-5% Metaphor agarose (BioWhittaker, Walkersville, MD). Products analyzed for SSCP were amplified with ^{33}P-dCTP and separated on denaturing acrylamide gels at 40W and 4ºC, with a Sequi-Gen GT System (Bio-Rad, Hercules, CA).

Immunization protocols. Mice studied in cytokine production assays were primed with KLH (Calbiochem, La Jolla, CA) in complete Freund's adjuvant (CFA) (DeKruyff et al. *J Immunol* **149**, 3468-76 (1992)). For measurement of airway hyperreactivity, mice were immunized with OVA intraperitoneally (i.p., 50 μg) complexed with aluminum potassium sulfate (alum) on day 0, and intranasally (i.n. 50 μg OVA in 50 μl of PBS) after light anesthesia on days 7, 8 and 9. Control mice received i.p. injections of alum alone and intranasal PBS. Airway hyperreactivity to inhaled methacholine was measured 24 hours after the last intranasal dose of OVA (day 10).

Measurement of Airway Responsiveness. Airway responses were assessed by methacholine-induced airflow obstruction from conscious mice placed in a whole body plethysmograph (Buxco Electronics Inc., Troy, NY), as described previously (Hansen et al. *J Clin Invest* **103**, 175-83 (1999)).

Cell Culture. Lymph node cells from mice primed with KLH were prepared as described previously (Yeung et al. *J Immunol* **161**, 4146-52 (1998)). Transgenic DO11.10 CD4 T cells were positively selected using MACS columns following incubation with anti-CD4 magnetic beads (Miltenyi Biotech, Germany). 2×10^4 cells/well were cocultured in 96-well round bottom plates with 250 μg/ml OVA and 1×10^4 bone marrow-derived dendritic cells/well. After seven days, the DO11.10 T cells were washed and restimulated with fresh antigen presenting cells and antigen at the concentration indicated. Antigen concentration for the primary DO11.10 cultures was titrated during the restimulation. Bone marrow–derived dendritic cells were generated as previously described with some modifications; 5×10^6 bone marrow cells were cultured in 9-cm diameter tissue culture dishes with 10 ml culture medium containing 20-25 U/ml GM-CSF. Loosely adherent cells were transferred onto a second dish on the sixth day of culture; within four days, these transferred cells were used as a source of dendritic cells.

Cytokine ELISA. ELISAs were performed as previously described in Macaulay et al. J Immunol 160, 1694-700 (1998); and Macaulay et al. J Immunol 158, 4171-9 (1997).

Monoclonal Antibodies. Monoclonal antibodies for ELISA and FACS analysis were purified from ascites fluid by ammonium sulfate precipitation and ion-exchange chromatography. Anti-clonotypic antibody KJ1-26.1, was generously provided by Dr. Philippa Marrack, National Jewish Medical Center, and the antibody was FITC-conjugated according to standard protocols prior to FACS.

Example 2

Identification of Human Tim Sequences

The positional cloning of the TIM gene family within a locus that confers protection against the development of Th2 responses and allergen-induced airway hyperreactivity provides an opportunity to greatly improve our understanding of the regulation of Th2 driven responses and atopic diseases. In addition, TIM-3 is specifically expressed on murine Th1 cells and anti-TIM-3 mAb leads to increased severity of experimental autoimmune encephalomyelitis (EAE). This emphasizes the importance of the gene family in T helper subset regulation.

The human Tim cDNAs, which are the orthologues of murine Tim-3 and Tim-4 were cloned by PCR. The human orthologue of TIM-1 was cloned as HAVcr-1, the

cellular receptor for hepatitis A virus. The TIM family genes are immediately adjacent to each other on human chromosome 5, in the order TIM-4, TIM-1, TIM-3, with no intervening genes. There are TIM pseudogenes on chromosomes 12 and 19. The gene family members are only moderately related. The protein sequences and relationship among the Tim gene family are shown in Figure 7.

The cytoplasmic domains of TIM gene family members are the most conserved domain between mouse and human orthologues, e.g., 77% identity between the human and mouse TIM-3 cytoplasmic domains. In contrast, the whole TIM-3 is only 63% identical between human and mouse. Each TIM gene contains a distinct predicted tyrosine signaling motif. The cytoplasmic region of TIM-1 contains two tyrosine residues and includes a highly conserved tyrosine kinase phosphorylation motif, RAEDNIY. The expanded region, SRAEDNIYIVEDRP, contains a predicted site for Itk and EGF receptor phosphorylation. Itk is known to phosphorylate phospholipase C-γ (PLC-γ), and thereby trigger a cascade of signaling events that are involved in T cell activation and helper T cell differentiation. Furthermore, Itk signaling affects Th1/Th2 differentiation, and $Itk^{-/-}$ mice do not develop strong Th2 responses. EGF receptor kinase activity is associated with cell survival and resistance to cell death. Similarly, TIM-3 contains distinct, conserved tyrosine phosphorylation and SH2 binding motifs in the cytoplasmic domain. This suggests that the interaction of a TIM with its ligand will engage an intracellular signaling pathway and that each TIM will be distinct in this signaling.

The extracellular IgV domain of the TIM proteins also contains a predicted integrin-binding motif that is similar to the SVVYGLR motif of osteopontin that is involved in adhesion via alpha(9)beta(1), alpha(4)beta(1), and alpha(4)beta(7) inetgrins. TIM-1 transfected pre-B cells of the 300.19 line demonstrate a high degree of adhesion an increased survival in cell culture, as compared to non-transfected 300.19 cells. TIM-1 and TIM-2 transfected CHO cells also demonstrate enhanced survival compared to untransfected CHO cells. These results demonstrate that the TIM proteins regulate cell adhesion and death

Genetic polymorphisms in the human Tim1 and Tim3 genes. SNPs or nucleotide polymorphisms and deletions/insertions present in the human *Tim1* gene are identified. Because SNPs are extremely common in the genome, occurring every 300-600 base pairs, only the coding region of *Tim1* was analyzed. Moreover, genetic variations that are common are also likely to be important. Initially cDNA is sequenced from T cells taken from 30-40 individuals (60-80 chromosomes). Power calculations show that surveying target sequences in coding regions of 60 chromosomes will easily detect SNPs with a population frequency of greater than 1%, and having a more than 90% chance of detecting alleles with a population frequency of 5% or greater. Therefore, screening 30-40

individuals for sequence variations captures most of the common, functionally relevant, non-conservative, DNA variation present in a population.

Since DNA variants/SNPs in close physical proximity often show strong dependency relationships (i.e., linkage disequilibrium), it is determined if a group of DNA variants (SNP haplotypes) are inherited together, and determined if screening for only a portion of these SNPs will be sufficient for identifying the haplotype. Analysis of large regions of various chromosomes indicate that discrete haplotype blocks (of tens to hundreds of kilobases) are generally present, each with limited diversity punctuated by apparent sites of recombination. To find haplotypes, cDNA is sequenced and searched for combinations of sequence variations that are seen repeatedly in multiple individuals.

Peripheral blood mononuclear cells (PBMC) were from 38 donors, and were stimulated *in vitro* with PHA (7.5 μg/ml) for 24 and 72 hours, or with Concavalin A (2μg/ml) for 24 hours. PMA (20ng/ml) and Ionomycin (1μM) were added during the last six hours of stimulation. The cells were then harvested and the total RNA was extracted using Trizol reagent(Invitrogen). To obtain cDNA templates for sequencing, RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's protocol. The cDNA were used to PCR amplify the full length of *Tim* cDNA using Herculase Hot Start™ high fidelity polymerase (Stratagene). The PCR primers were: (SEQ ID NO:37) GTGTCTGACAGTGGCGTA (forward), (SEQ ID NO:38) TTTGCCCAGGCAGAACCA (forward), CCACCCAAGGTCACGACT (reverse), (SEQ ID NO:39) ATGCCACGGACTAAGACC (reverse). The PCR products were purified with Qiagen QIAquick gel extraction reagents, and sequenced using four internal sequencing primers for *Tim1* and two internal sequencing primers for *Tim3*.

The full length *Tim1* RT-PCR product was cloned in these individuals by taking total RNA from activated T cells and transcribing it with Superscript II and oligo dT. *Tim1* cDNA was amplified with Expand high fidelity polymerase (Roche) to generate a 1 kb product spanning the *Tim1* coding region, which was purified with a PCR Purification kit (Invitrogen). This purified product was then cloned into the TOPO pEF6 vector (Invitrogen), followed by transformation of TOP10 competent bacteria. Bacterial colonies were grown on LB plates with ampicillin selection. Single colonies were picked and plasmid preps generated using Qiagen mini prep kits. Restriction mapping using Hind III digestion was used to select plasmids containing inserts in the correct orientation. These plasmids were then sequenced with three different primers, forward (T7), internal and reverse (BGH), and the sequences aligned in SeqMan program with NCBI human TIM reference sequence.

After sequencing *Tim1* from the chromosomes from 35 individuals (70 chromosomes) several polymorphisms in *Tim1* were identified, which are shown in Figure 8. These polymorphisms are numbered 1-7 (left column). The full sequence of human

TIM-1, which is listed in the NCBI database (NM_012206), is provided in Figure 8 as a reference point. This sequence is present in less than 20% of the chromosomes that were sequenced, due to the existence of multiple, prevalent sequence polymorphisms in the coding region. 6 additional sequence variations were identified, shown in Figure 8, and all of the polymorphisms were observed in the mucin, extracellular domain, as was true for mice, although the specific variations were distinct from those seen in mice. Importantly, there is a limited degree of association between these variants, in various combinations. The most pronounced variations are the insertion labeled polymorphism 1, 157insMTTTVP, which was observed in 65% of the chromosomes, and the deletion in polymorphism 5, 187ΔThr, was observed in 65% of the chromosomes. Polymorphism 4 was observed in 40% of the chromosomes, and the other polymorphisms were each observed in ≤5% of the chromosomes. Notably, most of these variations (2-6) are located within exon 3, the first mucin-encoding exon, and all of the variants occur at the genomic level and are not splice variants.

Based on this sequence analysis of mRNA, a more rapid method for analyzing the genomic DNA from the larger number of patients/controls has been developed. To screen individuals for the variations seen in sequences shown in Figure 8, the DNA is initially tested for simple sequence length polymorphisms (SSLP) in a 150 PCR product, which can detect the major insertion, polymorphism 1, and the deletion, polymorphism 5.

In addition, to genotype the other polymorphisms (2-4, 6, and 7) and identify novel polymorphisms, a relatively simple assay using single strand conformational polymorphism (SSCP) analysis of PCR products has been developed. Under well-optimized conditions, SSCP analysis detects more than 90% of single nucleotide substitutions and all length polymorphisms. For this analysis, PCR primers have been identified that amplify each exon of the *Tim* genes, and variants can be distinguished using standard non-denaturing SSCP gel electorphoresis methods (Figure 9). Non-denaturing polyacrylamide gel electrophoresis is used with an ABI 377 DNA sequence for high resolution SSCP analysis of each exon. Fluorescent end-labeled primers are synthesized and purified. Novel SSCP patterns that are detected during the high-throughput genotyping process will identify novel variants. Using this method, the genotype of patients and controls is rapidly analyzed.

The *Tim3* gene was analyzed using essentially the same methodologies. mRNA from activated T cells is sequenced to identify *Tim3* polymorphisms, as well as long range haplotypes between the Tim1 and Tim3. After sequencing *Tim3* cDNA representing 60 chromosomes, it has been found that *Tim3* is polymorphic, as it is in the mouse genome. However, only one polymorphism, Leu140Arg, is prevalent, found in

approximately 12% of the chromosomes represented.

Example 3

Expression of Tim Sequences

Murine TIM-3 protein is expressed on Th1 clones but not on naive T cells or Th2 cells. Using TCR transgenic T cells, TIM-3 protein was not expressed on Th1 cells after one or two rounds of Th1-directed differentiation but was expressed after the third and further rounds of Th1 stimulation. TIM-3 mRNA expression was detected somewhat earlier. In order to determine if TIM-3 gene expression was the same in human, TIM-3 and TIM-1 mRNA expression in human Th1 cells was examined using tetanus toxoid specific T cells generated by stimulation with antigen in the presence of IL-12 and anti IL-4 mAb. Given the association of TIM-1 with asthma, TIM-1 and TIM-3 mRNA expression in human Th2 cells was examined. Th2 cell lines were generated from allergic donors by *in vitro* stimulation with allergen, IL-4, and anti IL-12 mAb. RNA was analyzed by PCR for TIM gene expression.

TIM-3 was generally expressed after Th1 differentiation whereas TIM-1 was lost. Conversely, TIM-3 was not expressed in any of the Th2 but TIM-1 was expressed in all Th2 cells. Both TIM-1 and TIM-3 are expressed in monocyte-depleted, unstimulated peripheral blood mononuclear cells from the donors used to derive the Th1 and Th2 cell lines, presumably because this mixed population contains both Th1 and Th2 memory cells. These results suggest a reciprocal relationship with TIM-1 being expressed in Th2 and TIM-3 in Th1. This reciprocal relationship between TIM-1 and TIM-3 has also been observed in the mouse.

In human tissues, a 4.4 kb TIM-1 mRNA was very strongly expressed in kidney and testis. The 4.4-kb mRNA was present in almost all tissues, though it was faint in most. A 5.5-kb band was observed in colon and liver. A 7.5-kb band was observed in spleen, thymus, and peripheral blood leukocytes, and smaller than 4.4-kb bands were observed in some organs. These results suggest that hTIM-1 is expressed at some level in most human tissues and that a message of 7.5-kb may code for hTIM-1 in tissues of immunological interest. However, expression of Kim-1 (Kidney Injury Molecule-1), the rat homologue of TIM-1, increases in kidney upon ischemic injury. Since the MTN blots used in the expression analysis were prepared from mRNA extracted from cadavers, the increased expression of TIM-1 in kidney was re-analyzed. TIM-1 was not found to be overexpressed in kidney RNA obtained from normal kidney biopsies. Therefore, it is likely that the high levels of expression of TIM-1 observed in kidney and testis were due to an up-regulation in the expression of TIM-1 resulting from tissue injury. The injured kidney may express proteins that direct incoming inflammatory cells towards a more protective Th2

response rather than a destructive Th1 response.

Example 4

TIM Ligands and Antibodies

Generation of Antibodies. Generation of monoclonal antibodies against mouse TIM-1 allows examination of the cell surface expression of TIM-1 in different tissues, cell lines and mouse strains. Both alleles of mouse TIM-1 have been cloned into a vector for high protein expression (Invitrogen, pEF6-TOPO). Rats have been immunized and boosted with both Tim1 cDNA constructs to rapidly generate antibodies against cell surface molecules. This method with cDNA vaccination favors the production of mAb against cell surface epitopes since the Tim1 cDNA will be taken up by APC, which will express the TIM-1 as a cell surface molecule. In order to generate mAb that would bind equally well to both the BALB/c and the HBA TIM-1 (by binding to conserved domains of TIM-1 such as the Immunoglobulin domain of TIM-1), both the BALB/c and HBA Tim1 cDNA (pEF6-mTIMbalb and pEF6-mTIMhba) were injected into each rat.

Further boosting of the Tim1 cDNA-immunized rats was done with CHO cells stably transfected with the pEF6-mTIM-1-GFP expression constructs. CHO transfectants expressing high levels of mouse TIM-1 were sorted by FACS, and injected into the rats. Another mTIM-1 expressing cell was generated by stably transfecting the pre-B cell line 300.19 with the pEF6-mTIM-1 expression constructs. This line is used to screen the rat serum and the hybridomas following fusion for anti-TIM-1 antibody by flow cytometry. Rats have been generated which have high polyclonal titers against anti-TIM-1, as detected by the binding of rat serum (and a secondary FITC-goat anti-rat Ig) to stable pEF6-mTIM1-transfected 300.19 cells, as compared with control serum from unimmunized rats. This staining is specific for TIM-1 since there is no reactivity with nontransfected cells or cells transfected with TIM-2.

The rat spleen is removed and the splenocytes fused with a myeloma cell line (SP/2) to produce hybridomas. Hybridoma supernatants are screened using the TIM-1 transfected 300.19 cell lines to identify hybridoma clones that produce monoclonal anti-TIM-1. Specificity of the mAb for TIM-1 (and not other TIM proteins) is confirmed using TIM-2 transfected cells and mTIM-3 transfected cells or TIM-3 Ig fusion protein.

Antibody Staining. Th1 and Th2 cell lines were generated from both BALB/c and HBA DO11.10 spleen cells. RT-PCR for TIM-1 mRNA expression demonstrated that TIM-1 is expressed in Th2 lines, but not in Th1 lines, following two rounds of restimulation with antigen under standard polarizing conditions. DO11.10 T cells following two rounds of stimulation with antigen/APC under Th2 polarizing conditions were stained with the

polyclonal rat anti-TIM-1 antiserum. These Th2 cells expressed high levels of TIM-1.

These experiments showing preferential expression of TIM-1 in Th2 lines are quantified and confirmed using anti-Tim-1 mAbs and Northern blots. DO11.10 cells from BALB and HBA are cultured with antigen and APC, and restimulated for 1, 2, and 3 weeks under standard polarizing conditions (anti-IL-12 plus IL-4 or anti-IL-4 plus IL-12). After each week of stimulation, cells are stained with anti-TIM-1 mAb. By harvesting stimulated cells at various time points the kinetics of TIM-1 expression on T cells undergoing differentiation to Th1 or Th2 subset is determined. To determine if Tim-1 surface expression changes following T cell activation, we will also compare TIM-1 expression on resting and activated T cells one week after each round of antigen stimulation, by stimulating some cells with PMA and ionomycin. Activated cells are stained for intracellular cytokine expression to verify the Th subset differentiation of the T cells. Alternatively, quantitative RT-PCR or northern blots using mRNA harvested from T cells activated with PMA plus ionomycin, following each round of stimulation, are used to determine relative levels of mRNA production.

TIM-1-Ig fusion proteins BALB/c TIM-1-mIgG2a has been prepared, which is a fusion protein between the TIM-1 polypeptide and the Fc region of mouse immunoglobulin. The vector has been engineered to contain a mutation in murine IgG2a Fc that minimizes binding to Fc receptors. The TIM-1 fusion protein is utilized in characterization of TIM-1 function. The TIM-1 Ig fusion protein is expected to block TIM-1 function by binding to the TIM-1 ligand and interrupt TIM-1/TIM-1-ligand interactions.

Purified D1muc-Fc fusion protein containing the cys-rich immunoglobulin domain and 2/3 of the mucin-like region of TIM-1 fused to the hinge and Fc fragment of human IgG1 (IgVmuc-hIg) was run on a gel. This protein was expressed in CHO cells, and the IgVmuc-hIg protein was purified from CHO supernatants with protein-A agarose columns. Purified IgVmuc-hIg fusion protein neutralizes about 2 logs of HAV infectivity. In addition, treatment of HAV with IgVmuc-hIg produced a major shift in the sedimentation of the HAV particles, indicating that IgVmuc-hIg induced uncoating of the viral genome, whereas a fusion protein containing only the Ig-like region without the mucin domain (IgV-hIg) did not. This HAV neutralization system and the IgVmuc-hIg fusion protein will be used to analyze the function of TIM-1/HAV receptor alleles.

Based upon the *in vivo* effect of anti-TIM-3 mAb on macrophage expansion and activation, it was hypothesized that the TIM-3 ligand would be expressed on cells of the myeloid lineage. Dendritic cells (DC) were prepared from blood monocytes according to established protocols with 1000 U/ml IL-4 and 800 U/ml GM-CSF. DC were matured by replating the cells for 2 days in IL-4 (1000 U/ml) and GM-CSF (800 U/ml) supplemented

with IL-1β (10 ng/ml), TNF-α (10 ng/ml), IL-6 (1000 U/ml), and PGE_2 (1 μg/ml). Mature DCs stained positively with hTIM-3-Ig, though there was variability among donors, suggesting that mature DC express a ligand for the IgV domain of TIM-3. Bone marrow derived endothelial cells stained very weakly and B cell lines did not stain with TIM-3–Ig.

Although the intracytoplasmic tail of Tim1/huhavcr-1 is relatively short, it contains a sequence that is highly conserved between mouse, rat, human and monkey (RAEDNIYI), and which may be phosphorylated, and signal through interaction with other signal transduction molecules. The most likely candidate molecule that can bind the RAEDNIYI motif in T cells is the tyrosine kinase Itk. The interleukin-2 inducible tyrosine kinase, Itk is a nonreceptor protein tyrosine kinase of the Tec family that participates in the intracellular signaling events leading to T cell activation. Tec family members contain the conserved SH3, SH2, and catalytic domains common to many kinase families, but they are distinguished by unique sequences outside of this region. It is known that Itk phosphorylate phospholipase C-γ (PLC-γ), and triggers a cascade of signaling events that are involved in T cell activation and helper T cell differentiation. In the absence of Itk signaling, Th2 cells do not develop. These results suggest that TIM-1/huhavcr-1 may signal through Itk, thereby altering the cytokine development in CD4 T cells.

Example 5

TIM Knockout Mice

A knockout construct is used in the generation of a *Tim1* deficient mouse, which enables analysis of development of immune responses in the absence of TIM-1 molecules. In another approach, TIM-1-Ig fusion proteins or anti-TIM-1 monoclonal antibody are used to block the function of TIM-1 in $Tim1^{+/+}$ (wild type) mice. The Tim1 knockout mice and the anti-TIM-1 mAb approaches are complementary for the evaluation of the role of TIM-1 in T cell differentiation and in asthma pathogenesis.

The HBA mouse genomic sequence of *Tim1* has a deletion of exon 4, compared to the BALB/c sequence, due to an integrated L1 retroviral element at exon 4. If truncation of one exon reduces TIM-1 function, then total deletion of TIM-1 function by generation of Tim1 knockout mice, should severely limit the capacity of such mice to generate Th2 responses. TIM-1 KO mice are generated by deleting the TIM-1 exons 1 and 2, using cre-lox technology and BALB/c ES cells, which should eliminate cell surface expression of TIM-1, thereby demonstrating the importance of TIM-1 function in the development of Th2 cells and AHR.

To create an appropriate targeting construct, specific BACs containing members of the TIM gene family were identified by screening a C57/Bl6 BAC library (RPCI-

23) using high density filter sets. These BAC clones were used to build a 500 kB contig and physical map that covers the approximately 350 kb genomic region encompassing the *Tim* gene family. One specific BAC, RPCI-23-222F8, that contains the complete *Tim1* gene was chosen to generate a TIM targeting construct. The targeting construct deletes a 4 kb region encompassing the promotor region, signal exon, and IgV exon (exons 1 and 2), via homologous recombination with 5' and 3' arms flanking this region. This targeting vector can be used in either C57Bl/6 or BALB/c ES cells, since the homologous arms of the targeting construct are homologous to both BALB/c and HBA (C57Bl/6) DNA. The construct is introduced into ES cells, which are screened for the targeted allele by PCR and by Southern blots.

The targeting vector, pLOX, contains three loxP sites, and by expressing cre recombinase in the targeted ES cells, recombination will generate three forms of the targeted region, of which two alleles, A and B, are used to create *Tim1* KO mice. Selected ES cell clones are introduced into blastocysts to generate chimeric mice and bred for germline transmission of the knockout. Tail DNAs are analyzed for transmission of the knockout. Allele A is used initially, in which cre/lox recombination has used the outermost loxP sites to delete the TK and neomycin selection cassettes in addition to TIM-1 exons 1 and 2. The removal of the entire neo and TK cassette from the TIM-1 genomic targeting region prevents confounding artifacts that can arise secondary to the transcription of neo in close proximity to other genes of the same family. Alternatively, if allele A produces a lethal or confounding phenotype, mice are generated with targeted allele B, in which the neo and TK cassette has been deleted and the TIM-1 region is flanked by lox-p sites. Use of this conditional targeting approach should allow T cell-specific deletion of Tim-1 in mice expressing cre-recombinase under the control of a T cell specific promoter.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising a mammalian TIM gene sequence.

2. An isolated nucleic acid molecule according to Claim 1, wherein said mammalian TIM gene sequence is a human sequence selected from the group consisting of alleles 1, 2, 3, 4, 5, 6, and 7 of TIM-1, TIM-3, and TIM-4.

3. An isolated nucleic acid according to Claim 1, wherein said mammalian TIM gene sequence is a mouse sequence selected from the group consisting of TIM-1, TIM-2, TIM-3, and TIM-4.

4. An isolated nucleic acid comprising at least 20 nucleotides of contiguous sequence as set forth in any one of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34 or 36.

5. An isolated nucleic acid comprising a sequence as set forth in any one of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34 or 36.

6. An isolated nucleic acid molecule according to Claim 1, comprising a probe for detection of a TIM gene polymorphism.

7. An array of oligonucleotides comprising:
two or more probes according to Claim 6.

8. A cell comprising a nucleic acid composition according to claim 1.

9. A purified polypeptide composition comprising at least 50 weight % of the protein present as the product of the nucleic acid of Claim 1 or a fragment thereof.

10. The purified polypeptide according to Claim 9, wherein said polypeptide comprises a sequence as set forth in SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33 and 35.

11. An antibody specific for a mammalian TIM polypeptide.

12. A polypeptide molecule which is a fusion protein comprising a TIM protein or fragment thereof and an antibody Fc region.

13. The polypeptide according to Claim 12, wherein said TIM protein is human TIM-1, TIM-3 or TIM-4.

14. The polypeptide according to Claim 12, wherein said TIM protein is mouse TIM-1, TIM-2, TIM-3 or TIM-4.

15. The polypeptide according to Claims 12 to 14, wherein said Fc region is the Fc region of human or mouse immunoglobulin.

16. The polypeptide according to Claims 12 to 14, wherein the Fc region is the Fc region of mouse immunoglobulin.

17. A nucleic acid sequence encoding a polypeptide according to any one of Claims 12 to 16.

18. An expression vector, wherein said vector comprises a nucleic acid sequence encoding a polypeptide according to any one of Claims 12 to 16.

19. A cell, wherein said cell expresses a polypeptide according to any one of Claims 12 to 16.

20. A method for detecting a predisposition to asthma in an individual, the method comprising:

analyzing said individual for the presence of at least one predisposing TIM polymorphism, wherein the presence of said predisposing polymorphism is indicative of an increased susceptibility to asthma.

21. The method according to Claim 20, wherein said analyzing step comprises detection of specific binding between genomic DNA or mRNA of said individual with a probe or probes according to Claim 6.

22. The method according to Claim 20, wherein said analyzing step comprises detection of specific binding between cells or protein from said individual and the antibody according to Claim 11.

23. A non-human transgenic animal model for TIM gene function comprising a knockout of a TIM gene.

24. A method of screening for biologically active agents that modulate TIM function, the method comprising:

combining a candidate biologically active agent with any one of:

(a) a mammalian TIM polypeptide;

(b) a cell comprising a nucleic acid encoding a mammalian TIM polypeptide;

(c) a non-human transgenic animal model for TIM gene function; and determining the effect of said agent on TIM function.

25. The method according to Claim 24, wherein said biologically active agent downregulates or upregulates TIM expression.

26. The method according to Claim 24, wherein said biologically active agent inhibits or increases activity of said TIM polypeptide.

27. A method for the diagnosis or staging of immune dysfunction in an individual, the method comprising:

analyzing a sample of said individual for the presence of at least one predisposing TIM polymorphism; wherein the presence of said predisposing polymorphism is indicative of an increased susceptibility to said immune dysfunction.

28. A method for the diagnosis or staging of cancer in an individual, the method comprising:

analyzing a sample of said individual for the presence of at least one predisposing TIM polymorphism; wherein the presence of said predisposing polymorphism is indicative of an increased resistance to cancer therapy.

29. A method for the diagnosis or staging of cancer in an individual, the method comprising:

analyzing a sample of said individual for the presence of at least one predisposing TIM-1 expression profile; wherein the expression profile of TIM-1 indicative of an increased resistance to cancer therapy.

30. A method for the diagnosis or staging of cancer in an individual, the method comprising:

analyzing a sample of said individual for the presence of at least one predisposing TIM-4 expression profile; wherein the expression profile of TIM-4 indicative of an increased resistance to cancer therapy.

31. Use of an agent that modulates function of a TIM polypeptide for the preparation of a pharmaceutical composition for treatment of an immunological disorder.

32. The use according to Claim 31, wherein said TIM polypeptide is TIM-1, TIM-3 or TIM-4.

33. The use according to Claim 31, wherein said agent is a fusion protein according to any one of Claims 12 to 16.

34. The use according to Claim 31, wherein said agent is an antibody or antibody fragment specific for TIM-1, TIM-3 or TIM-4.

35. The use according to Claim 31, wherein said agent is a compound that inhibits, the function of TIM-1, TIM-3 or TIM-4.

36. The use according to any one of Claims 31 to 35, wherein said immunological disorder is asthma.

37. The use according to any one of Claims 31 to 35, wherein said immunological disorder is an allergy.

38. The use according to any one of Claims 31 to 35, wherein said immunological disorder is eczema.

39. The use according to any one of Claims 31 to 35, wherein said immunological disorder is an autoimmune disease.

40. The use according to any one of Claims 31 to 35, wherein said immunological disorder is an atopic disease.

41. The use of an agent that modulates function of a TIM polypeptide for the preparation of a pharmaceutical composition for the treatment of malignancy.

42. The use according to Claim 41, wherein said TIM polypeptide is TIM-1, TIM-3 or TIM-4.

43. The use according to Claim 41, wherein said agent is a fusion protein according to any one of Claims 12 to 16.

44. The use according to Claim 41, wherein said agent is an antibody or antibody fragment specific for TIM-1, TIM-3 or TIM-4.

45. The use according to Claim 41, wherein said agent is a compound that inhibits the function of TIM-1, TIM-3 or TIM-4 and potentiates the effect of chemotherapeutic agents or radiation therapy.

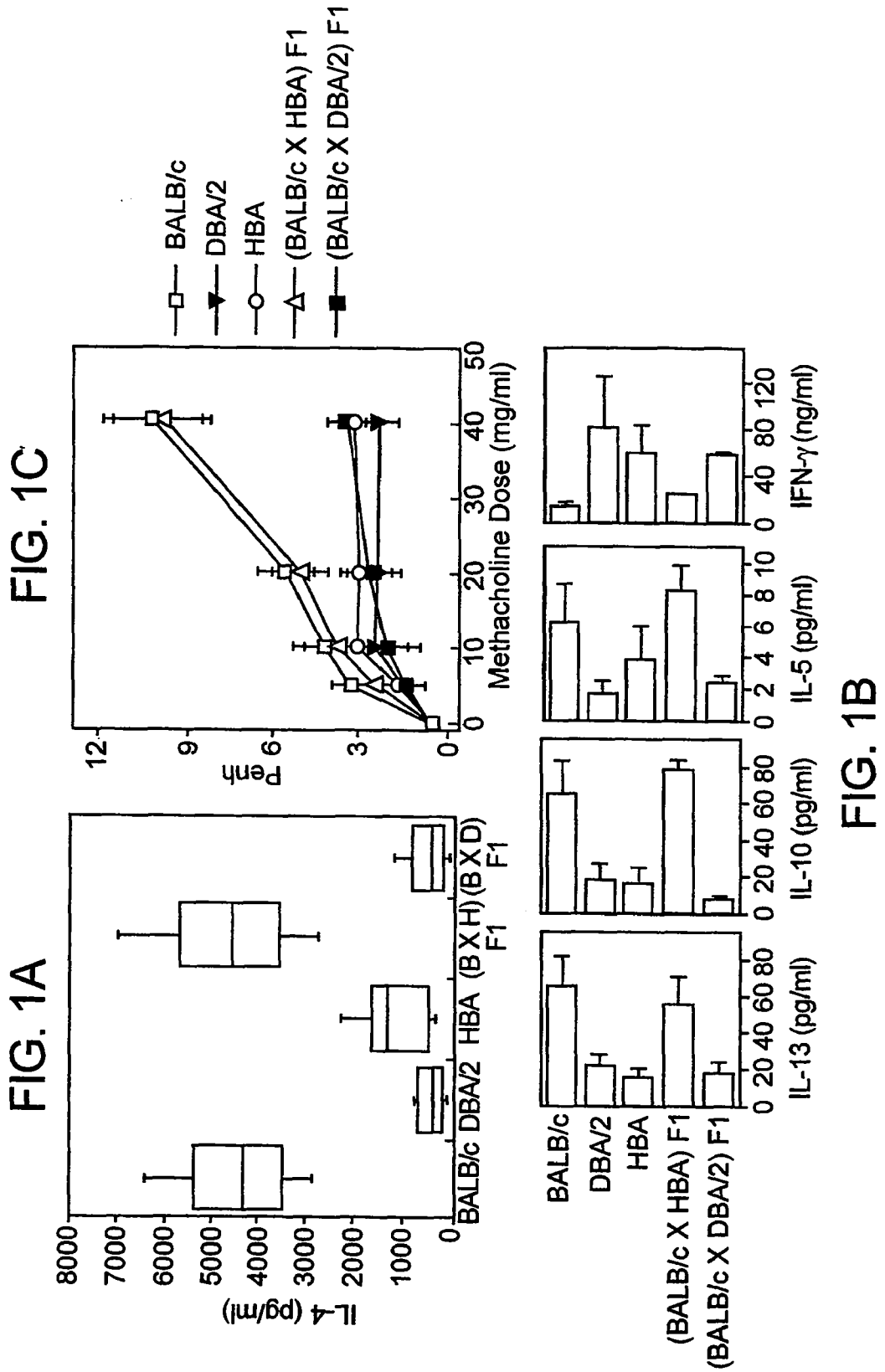
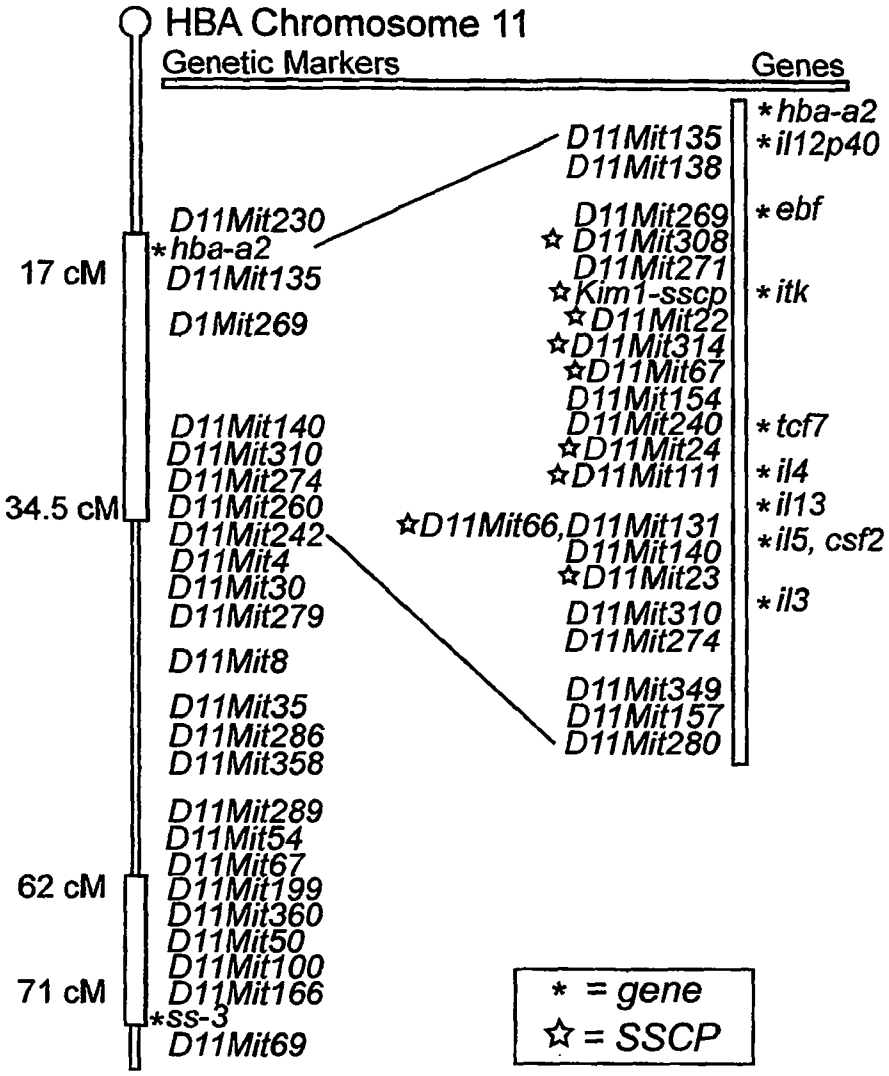


FIG. 1A
IL-4 (pg/ml)
8000
7000
6000
5000
4000
3000
2000
1000
0
BALB/c
DBA/2
HBA
(B X H) F1
(B X D) F1
FIG. 1C
Penh
12
9
6
3
0
0
10
20
30
40
50
Methacholine Dose (mg/ml)
BALB/c
DBA/2
HBA
(BALB/c X HBA) F1
(BALB/c X DBA/2) F1
FIG. 1B
IL-13 (pg/ml)
0 20 40 60 80
IL-10 (pg/ml)
0 20 40 60 80
IL-5 (pg/ml)
0 2 4 6 8 10
IFN-γ (ng/ml)
0 40 80 120


HBA Chromosome 11
Genetic Markers
Genes
17 cM
34.5 cM
62 cM
71 cM
D11Mit230
*hba-a2
D11Mit135
D1Mit269
D11Mit140
D11Mit310
D11Mit274
D11Mit260
D11Mit242
D11Mit4
D11Mit30
D11Mit279
D11Mit8
D11Mit35
D11Mit286
D11Mit358
D11Mit289
D11Mit54
D11Mit67
D11Mit199
D11Mit360
D11Mit50
D11Mit100
D11Mit166
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D11Mit135
D11Mit138
D11Mit269
☆D11Mit308
D11Mit271
☆Kim1-sscp
☆D11Mit22
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D11Mit154
D11Mit240
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D11Mit140
☆D11Mit23
D11Mit310
D11Mit274
D11Mit349
D11Mit157
D11Mit280
*hba-a2
*il12p40
*ebf
*itk
*tcf7
*il4
*il13
*il5, csf2
*il3
* = gene
☆ = SSCP

FIG. 2

FIG. 3A

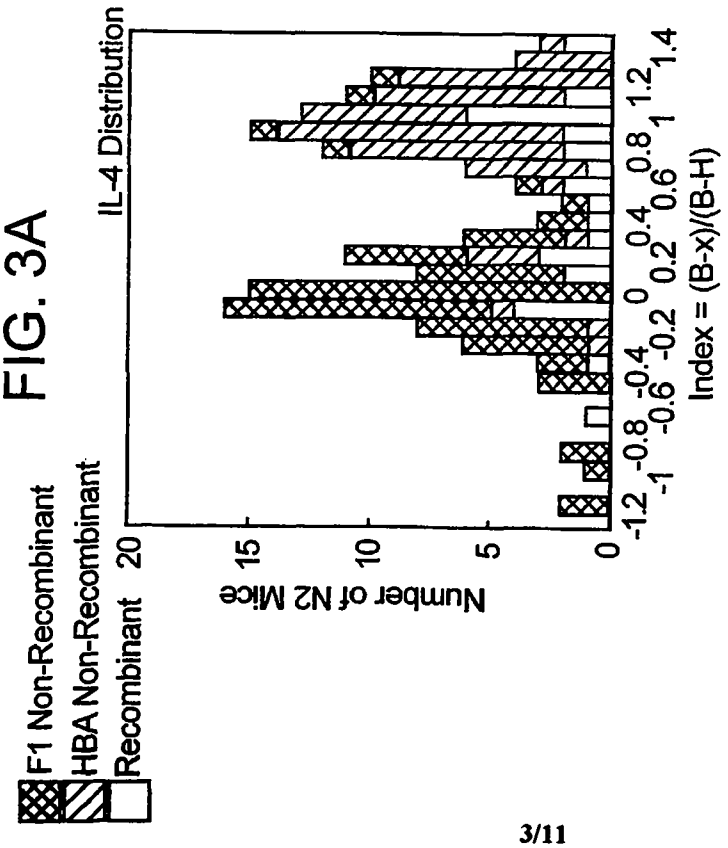

FIG. 3B

FIG. 3C

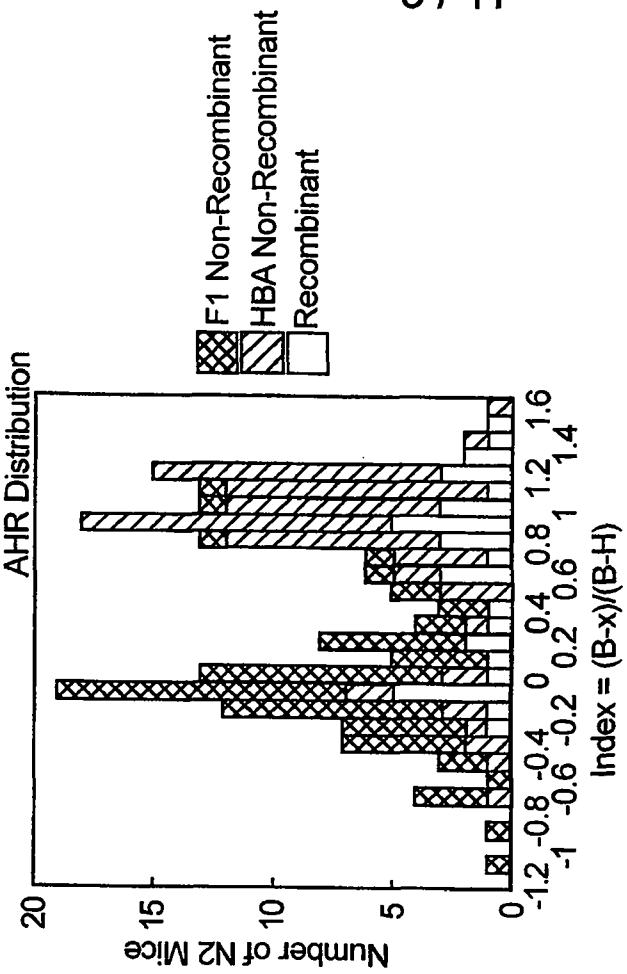

FIG. 3D

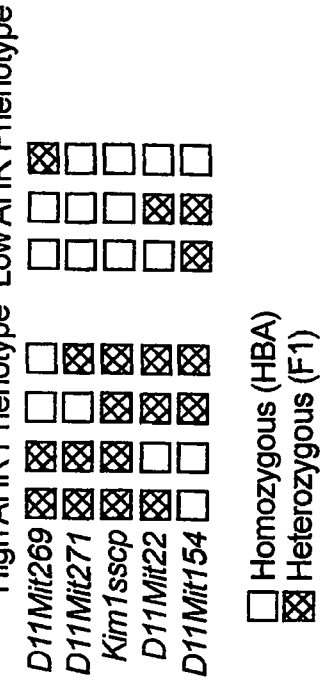

FIG. 4

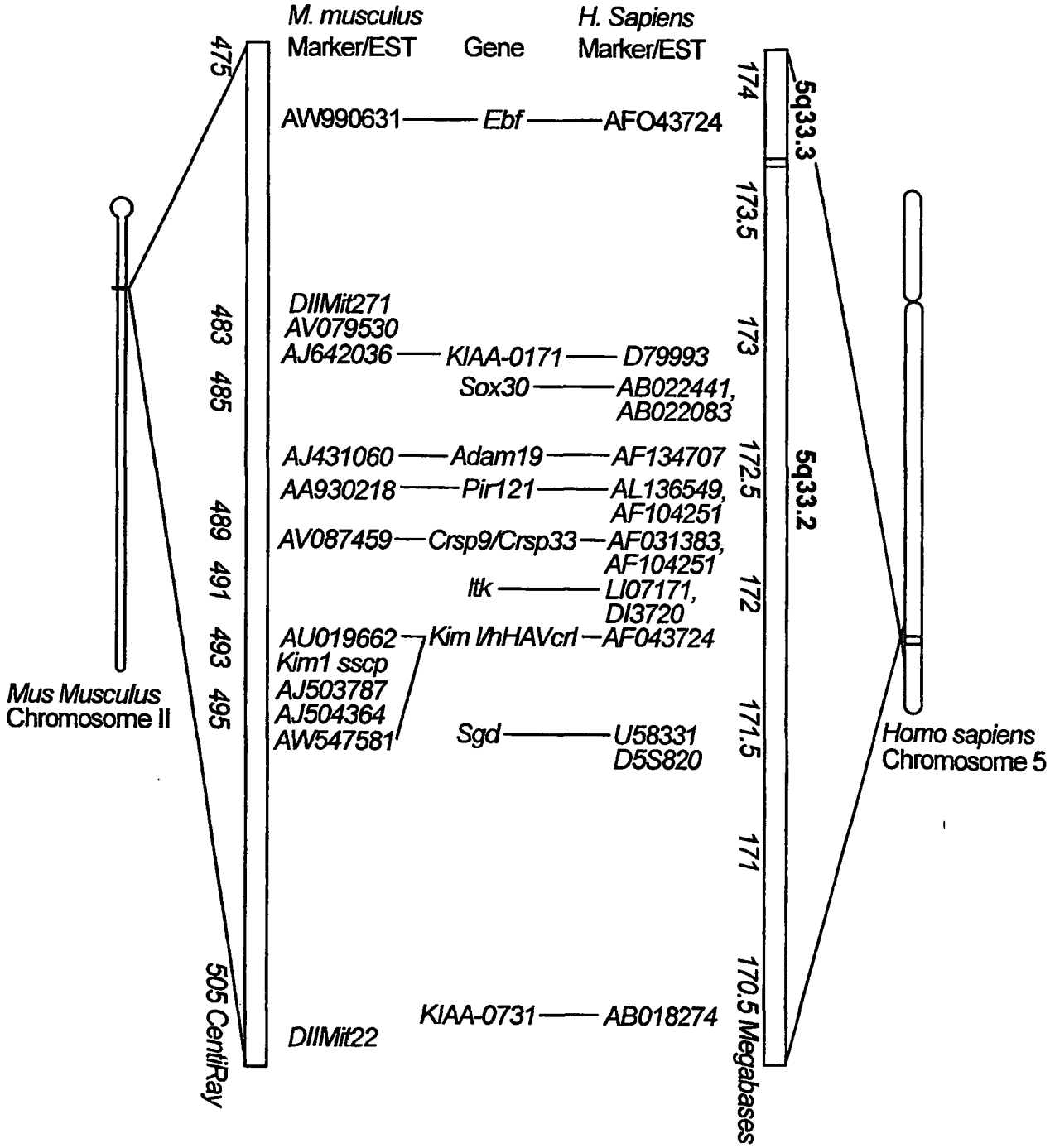
M. musculus
Marker/EST
Gene
H. Sapiens
Marker/EST
475
AW990631
Ebf
AFO43724
174
5q33.3
173.5
DIIMit271
AV079530
483
AJ642036
KIAA-0171
D79993
173
Sox30
AB022441,
AB022083
485
AJ431060
Adam19
AF134707
172.5
5q33.2
AA930218
Pir121
AL136549,
AF104251
489
AV087459
Crsp9/Crsp33
AF031383,
AF104251
491
Itk
LI07171,
DI3720
172
AU019662
Kim I/hHAVcrl
AF043724
493
Kim1 sscp
AJ503787
AJ504364
AW547581
495
Sgd
U58331
D5S820
171.5
171
170.5 Megabases
505 CentiRay
KIAA-0731
AB018274
DIIMit22
Mus Musculus
Chromosome II
Homo sapiens
Chromosome 5

FIG. 5A

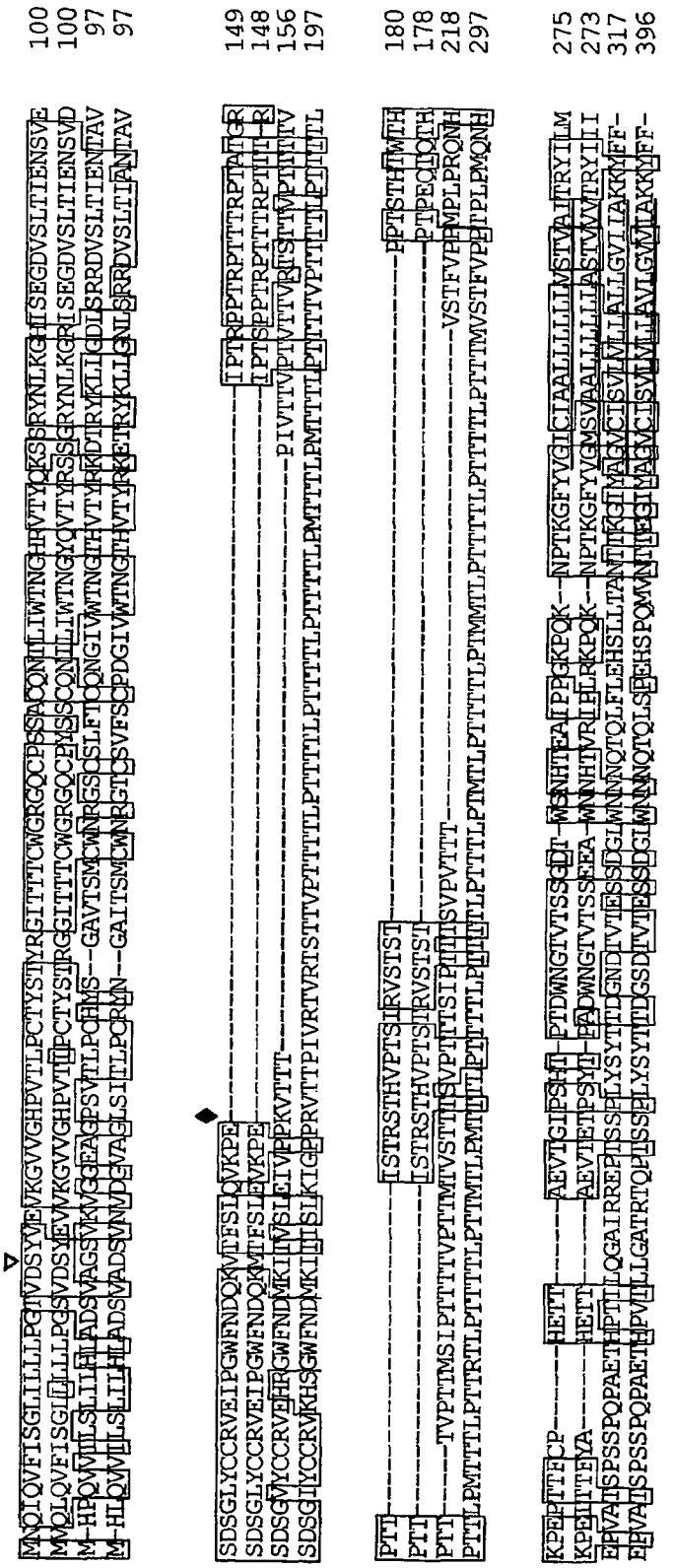

FIG. 5B

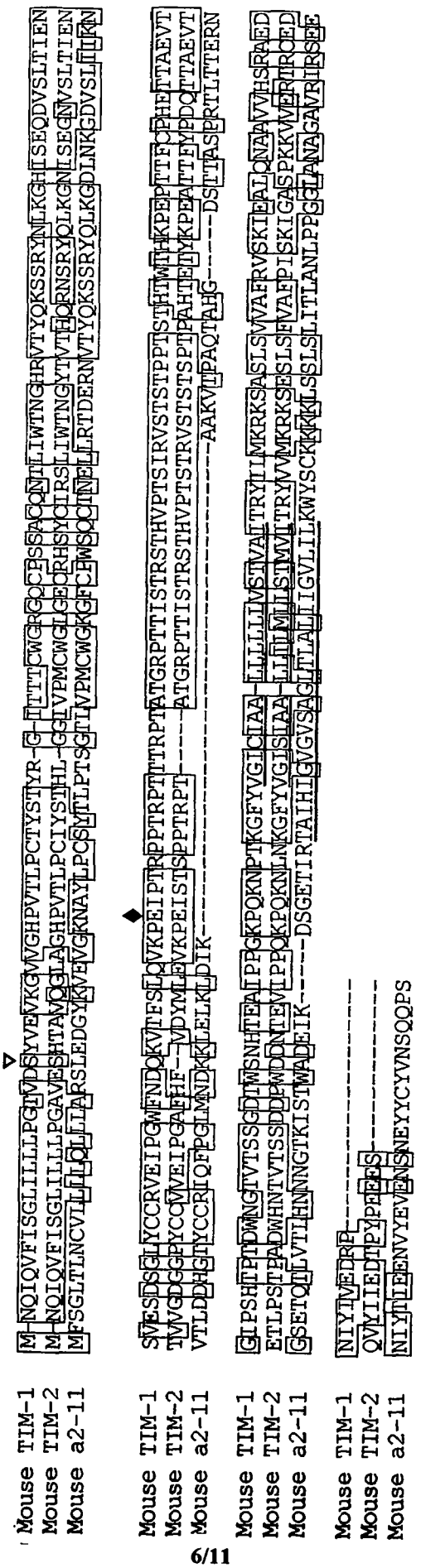

FIG. 5C

TIM-1 Variants

```
                                  ∇
1     MNQIQVFISGLILLLPGAVDSYVEVKGVVGHPVTLPCTYSTYRGITTTCWGRGQCPSSACQNTLIWTNGHRVTYQKSSRYNLKGHISEGDVSLTIENSVE   HBA
1     MNQIQVFISGLILLLPGTVDSYVEVKGVVGHPVTLPCTYSTYRGITTTCWGRGQCPSSACQNTLIWTNGHRVTYQKSSRYNLKGHISEGDVSLTIENSVE   BALB/c

                 ♦
100   SDSGLYCCRVEIPGWFNDQKVTFSLQVKPEIPTRPPRRPTTTRPTATGRPTTISTRSTHVPTSTRVSTSTPPTSTHTWTHKPEPTTFC----------   HBA
100   SDSGLYCCRVEIPGWFNDQKVTFSLQVKPEIPTRPPTRPTTTRPTATGRPTTISTRSTHVPTSIRVSTSTPPTSTHTWTHKPEPTTFCPHETTAEVTGIP   BALB/c

200   ---PTDWNGTVTSSGDTWSNHTEAIPPGKPQKNPTKGFYVGICIAALLLLLLVSTVAITRYILMKRKSASLSVVAFRVSKIEALQNAAVVHSRAEDNIYI   HBA
200   SHTPTDWNGTVTSSGDTWSNHTEAIPPGKPQKNPTKGFYVGICIAALLLLLLVSTVAITRYILMKRKSASLSVVAFRVSKIEALQNAAVVHSRAEDNIYI   BALB/c

300   VEDRP          HBA
300   VEDRP          BALB/c
```

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A2-11/TIM-3 Variants

```
                                  ∇
1     MFSGLTLNCVLLLLQLLLARSLENAYVFEVGKNAYLPCSYTLSTPGALVPMCWGKGFCPWSQCTNELLRTDERNVTYQKSSRYQLKGDLNKGDVSLIIKN   HBA
1     MFSGLTLNCVLLLLQLLLARSLEDGYKVEVGKNAYLPCSYTLPTSGTLVPMCWGKGFCPWSQCTNELLRTDERNVTYQKSSRYQLKGDLNKGDVSLIIKN   BALB/c

                                  ♦
100   VTLDDHGTYCCRIQFPGLMNDKKLELKLDIKAAKVTPAQTAHGDSTTASPRTLTTERNGSETQTLVTLHNNNGTKISTWADEIKDSGETIRTAIHIGVGV   HBA
100   VTLDDHGTYCCRIQFPGLMNDKKLELKLDIKAAKVTPAQTAHGDSTTASPRTLTTERNGSETQTLVTLHNNNGTKISTWADEIKDSGETIRTAIHIGVGV   BALB/c

200   SAGLTLALIIGVLILKWYSCKKKKLSSLSLITLANLPPGGLANAGAVRIRSEENIYTIEENVYEVENSNEYYCYVNSQQPS   HBA
200   SAGLTLALIIGVLILKWYSCKKKKLSSLSLITLANLPPGGLANAGAVRIRSEENIYTIEENVYEVENSNEYYCYVNSQQPS   BALB/C
```

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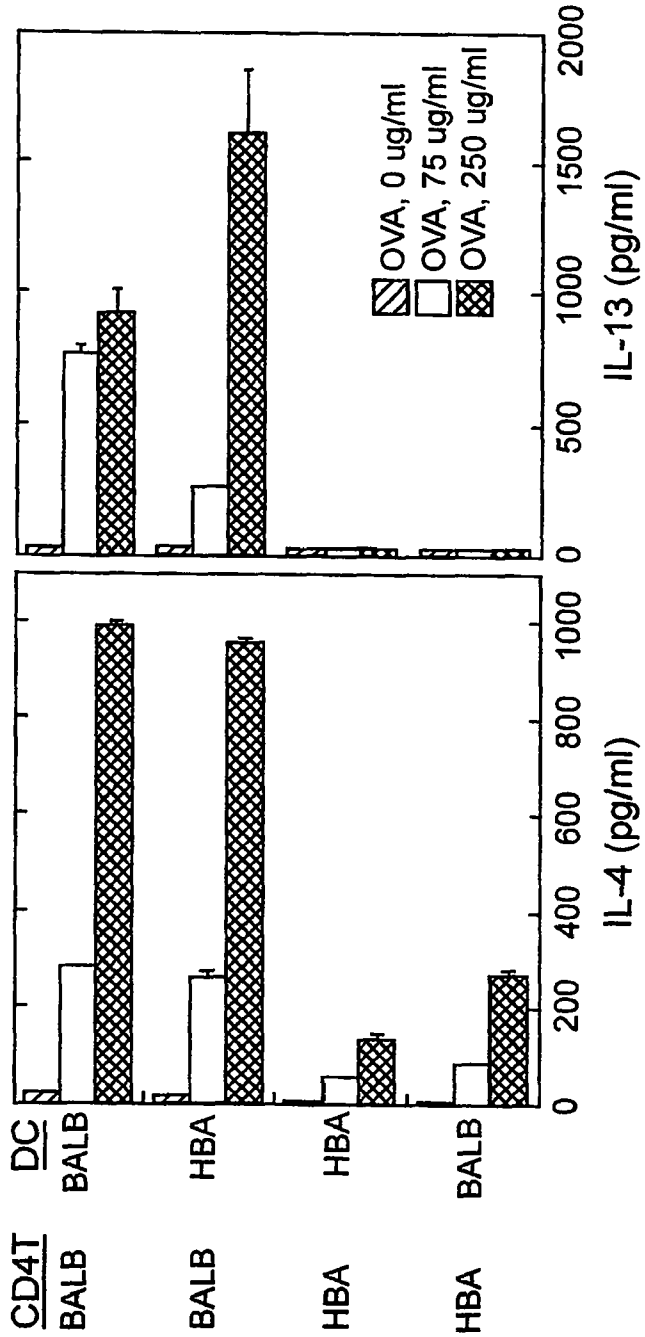
OVA, 0 ug/ml
OVA, 75 ug/ml
OVA, 250 ug/ml
IL-13 (pg/ml)
0
500
1000
1500
2000
IL-4 (pg/ml)
0
200
400
600
800
1000
DC
CD4T
BALB
BALB
HBA
HBA
HBA
HBA
BALB
BALB

FIG. 6

FIG. 7

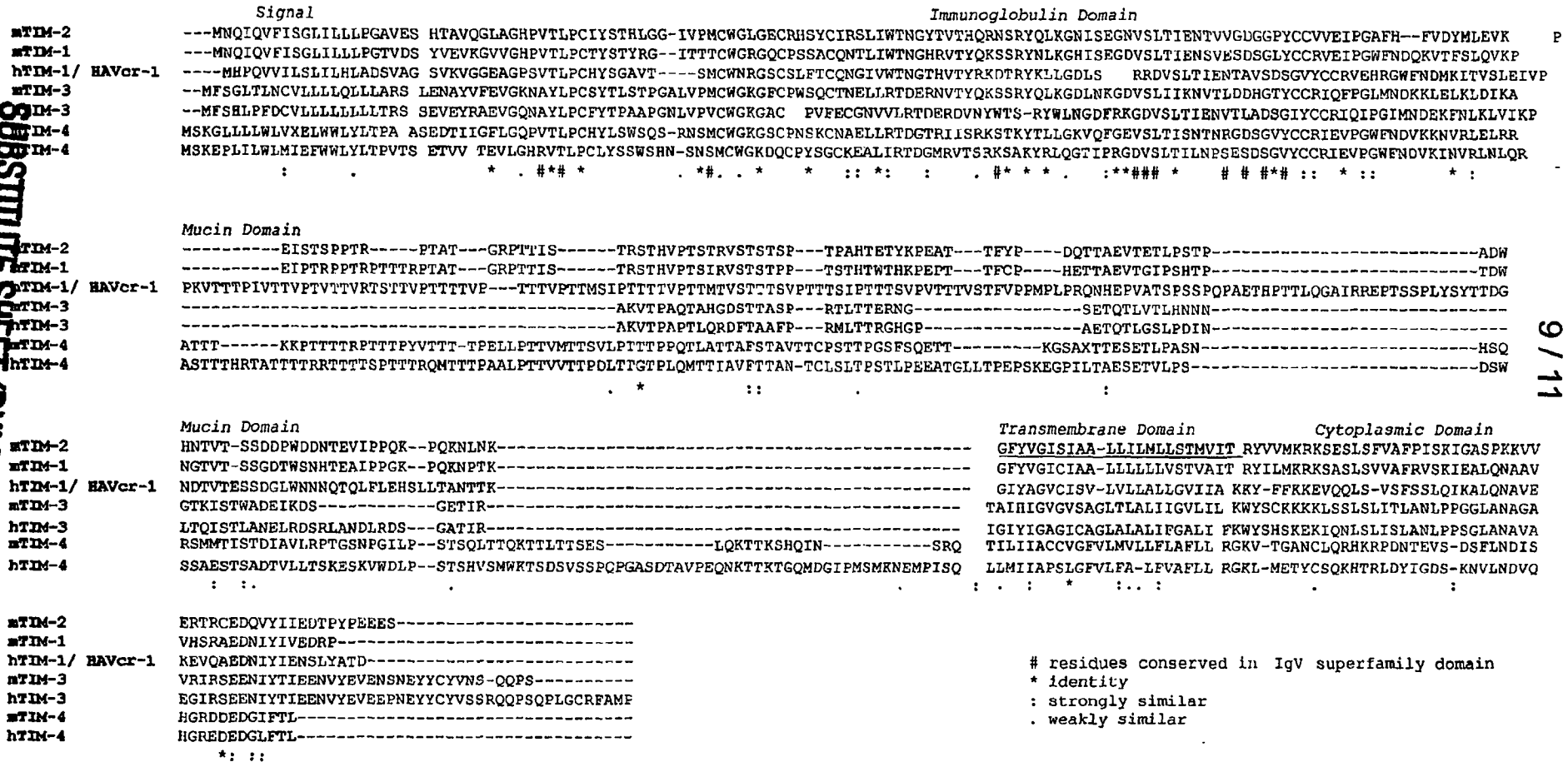

FIG. 8

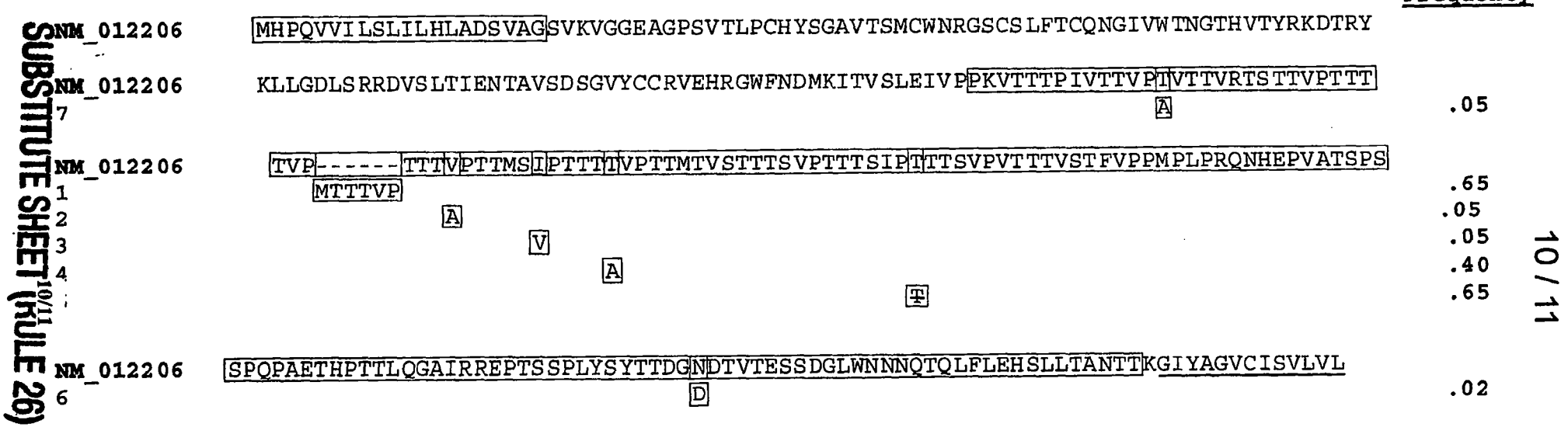

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FIG. 9