Doctoral Thesis

Generation and analysis of T1-deficient and T1-Fc-transgenic mice

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Generation and Analysis of T1-deficient and T1-Fc-transgenic Mice

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1. Summary

T1 (ST2) has been isolated originally as a growth factor and oncoprotein responsive gene. It encodes an orphan receptor belonging to the interleukin-1 receptor (IL-1R) family. Two different forms of the T1 protein exist: a membrane bound molecule with a cytoplasmic domain, whose predominate sites of expression are mast cells and Th2 cells and a soluble secreted protein lacking the transmembrane and cytoplasmic domains, that is produced by fibroblasts and osteoblasts. In these latter cells expression is strongly increased after stimulation with growth factors, proinflammatory cytokines and in response to oncogene expression.

Much research has been directed towards an understanding of T1 gene expression and regulation whereas little is known about the function of T1. In an attempt to elucidate the role of the T1 protein we generated two different mutant mouse strains that are either unable to synthesize T1 proteins or constitutively produce and secrete high levels of a T1-Fc fusion protein into the serum. It was anticipated that the sustained high amount of soluble T1 protein scavenges the putative T1 ligand and thereby prevents it from binding to the T1 receptor.

Since T1 is expressed on the surface of Th2 but not Th1 cells we focused our investigations on the role of this molecule in Th2-mediated immune responses. Previous studies, performed in other laboratories with anti-T1 monoclonal antibodies have suggested that T1 is critical for the development of normal Th2-type responses. As a model system to study Th2-mediated protective immunity we used infection of mice with the parasitic nematode *Nippostrongylus brasiliensis*. In this work we show that the protective immunity against *N. brasiliensis* was intact in both the T1-deficient and T1-Fc transgenic mice. However, we found that the amount of IL-5 producing Th2 cells isolated from infected lungs was slightly decreased compared to control animals. This decrease was reflected in the reduced eosinophil infiltration into the lung, and was especially evident in the T1-Fc transgenic animals. Studies using viral infection with vesicular stomatitis virus (VSV) and lymphocytic choriomeningitis virus (LCMV) revealed that antibody and CTL responses were not affected in the T1 mutant mice. Likewise, infection with the bacterium *Listeria monocytogenes* was normally cleared in T1 mutant mice and in some experiments, T1-deficient mice even displayed a lower bacterial burden in the target organs suggesting a slightly enhanced innate immune response in the absence of T1.

Taken together, we found that in the infection models of *N. brasiliensis*, LCMV, VSV and *L. monocytogenes* a normal protective immune response could develop in the complete absence of the T1 protein (T1-deficient mice) and in presence of a soluble form of T1 in the serum (T1-Fc transgenic mice).
2. Zusammenfassung

T1 (ST2) wurde ursprünglich als ein Gen isoliert, das durch Wachstumsfaktoren und Onkoproteine induziert wird. Es codiert für einen Rezeptor, der zur Familie der Interleukin-1 Rezeptoren (IL-1R) gehört. Es gibt zwei verschiedene Formen des T1 Proteins: ein membrangebundenes Molekül mit einer cytoplasmatischen Domäne, welches auf Mastzellen und Th2 Helferzellen exprimiert wird, sowie ein lösliches, sezerniertes Protein, das weder eine transmembranäre noch eine zytoplasmatische Domäne aufweist und von Fibroblasten sowie Osteoblasten in geringen Mengen hergestellt wird. Nach Stimulation mit Wachstumsfaktoren, pro-inflammatorischen Zytokinen oder bei Expression eines Onkogens wird die Menge an sezerniertem T1 Protein stark erhöht.

Während über die Expression und Regulation des T1 Gens einiges bekannt ist, weiss man sehr wenig über die Funktion des T1 Proteins. Um diese etwas näher zu untersuchen, haben wir zwei Mausmodelle etabliert: während die eine Maus das T1 Protein nicht mehr synthetisieren kann, produziert die andere Maus die lösliche Form von T1 als ein Fusionsprotein mit dem Fc Teil des humanen IgGs und gibt dieses in grossen Mengen ins Blut ab. Wir erwarten, dass das T1-Fc Protein den postulierten T1 Liganden bindet und verhindert, dass er an die Zielzellen binden kann.

Da T1 auf der Oberfläche von Th2 nicht aber von Th1 Zellen exprimiert wird, konzentrierten wir uns in dieser Arbeit auf die Rolle von T1 in der von Th2 Zellen dominierten Immunantwort. Ein Modellsystem dafür stellt eine Infektion von Mäusen mit dem Nematoden *Nippostrongylus brasiliensis* dar. In der vorliegenden Arbeit haben wir gezeigt, dass die schützende Immunantwort sowohl in T1-defizienten als auch in T1-Fc-transgenen Mäusen nicht beeinträchtigt ist. Wir konnten aber auch zeigen, dass die Menge an IL-5 produzierenden Th2 Zellen, die aus der Lunge von infizierten Tieren isoliert wurden, im Vergleich zu wildtyp Kontrolltieren geringer war. Diese Abnahme widerspiegelte sich in einer leichten Hemmung der eosinophilen Leukozyten-Infiltration in die Bronchoalveolar-Flüssigkeit, besonders bei den T1-Fc transgenen Tieren. Bei viralen Modellsystemen wie Infektion mit dem Vesikulären Stomatitis Virus (VSV) oder dem Lymphozytären Choriomeningitis Virus (LCMV) erwies sich die antivirale Immunantwort in T1-defizienten und T1-Fc transgenen Mäusen mit den wildtyp Kontrolltieren vergleichbar. Eine bakterielle Infektion mit *Listeria monocytogenes* wurde ebenfalls erfolgreich bekämpft, wobei in einigen Experimenten eine geringere Anzahl Listerien in den Zielorganen gefunden wurde, was auf eine erhöhte unspezifische Immunantwort in den T1-defizienten Mäusen hinweist. Insgesamt stellte sich heraus, dass sowohl in Abwesenheit des T1 Proteins als auch in Anwesenheit des löslichen T1-Fc Fusionsproteins im Serum bei den getesteten Infektionsmodellen eine schützende Immunantwort noch möglich ist.
3. Introduction

One major goal of today’s biomedical research is to sequence the entire genome of various organisms and to ascribe to every identified gene its linked function(s). Despite the extensive knowledge of DNA sequences, intron/exon structures, and regulatory sequences of many genes, we know very little or nothing about the functions of the proteins encoded by an increasingly large number of genes. One of them is the T1 gene, which was isolated originally in our laboratory as an oncoprotein responsive gene. Its regulation and expression has been extensively studied, and its translation products are known as well, but the biologic function of the T1 protein is still unknown. This study focuses on the physiologic role of the T1 protein.

3.1 Isolation and Characterization of the T1 Gene

The T1 gene was originally identified in mouse 3T3 fibroblastic cells in a search for genes that are transcriptionally activated upon the conditional expression of the oncogenes Ha-ras(EJ) and v-mos as well as by serum growth factors (1-3). Later it was again isolated in a screen for delayed early serum-responsive genes (4). Due to its cloning by different groups it has received several names: T1 (1, 3), ST2 (2), and DER4 (4). The human and rat homologs of the murine T1 have been cloned from human leukocytes (5) and as a Fos-responsive gene from rat fibroblasts (Fit-1), respectively (6). T1 is transcribed into an abundant 2.7 kb and a rare 5 kb mRNA upon stimulation of fibroblasts with proliferation-inducing agents (serum, platelet derived growth factor, lysophosphatidic acid, fibroblast growth factor, 12-O-tetradecanoylphorbol-13-acetate [TPA]) (7), with proinflammatory cytokines (interleukin-1 [IL-1], tumor necrosis factor alpha [TNF-α]) (8, 9), or in response to oncogene expression (1, 3). In fibroblasts both transcripts are initiated at the same site, but are differentially processed at the 3’ end. The 2.7 kb mRNA is polyadenylated at the site of exon 8, whereas the 5 kb mRNA is generated by ignoring this poly(A) signal. A splice site 24 bp upstream of the termination codon in exon 8 is used, placing the polyadenylation site several kb further downstream, thus leading to the inclusion of exons 9-11 (6, 10). In various human cells and tissues, three different messages (4.2, 2.7, and 1.4 kb) are expressed in varying amounts. The 1.4 kb transcript is uniquely found in human cells (8).
The short 2.7 kb mRNA encodes a secreted, heavily N-glycosylated protein of 337 amino acids (T1-S) with a hydrophobic amino-terminal leader peptide (1). The predicted signal peptidase cleavage site is at position 26. The mature protein has a predicted molecular weight of 38.5 kDa and its apparent molecular weight varies between 45-70 kDa, due to different extents of glycosylation in different cells (1, 11). Nine potential N-linked glycosylation sites are predicted from its amino acid sequence.

The 5-kb mRNA is translated into a receptor-like protein of 567 amino acids with a calculated molecular weight of 65 kDa (T1-M). The protein is also heavily glycosylated and migrates on SDS-PAGE with a molecular weight of 95-110 kDa (12).

The ectodomains of the two forms of T1 are identical at the amino terminus and differ only nine amino acids before the carboxy terminal end of the small protein. The large T1 protein consists of an additional transmembrane domain of 24 amino acids and a cytoplasmic domain of 201 amino acids (10). This membrane anchored form of T1 is predominantly expressed in primary mast cells and Th2 cells (13, 14). In mast cells transcription was shown to initiate 10.5 kb further upstream than in fibroblasts. Both of the alternative exon 1 used in mast cells and in fibroblasts are spliced to the common exon 2 where the ATG initiation codon is located (13) (Fig. 1).

**Figure 1. Structure of the T1 gene.**

Coding and non-coding exons are indicated by filled and open boxes. The enhancer used in fibroblasts and the region containing the regulatory elements for mast cell specific expression are indicated by an open circle and a diamond, respectively. The alternative first exons are spliced to exon 2 where translation initiation occurs. Exons 2-8 encode the extracellular domain, whereas the transmembrane and intracellular domains are encoded by exons 9-11. The splicing pattern in mast cells and fibroblasts is given above and below the line, respectively. The gene structure is taken from Gächter et al., Tominaga et al. (13, 15) and unpublished results.
The extracellular portion of the T1 proteins consists of three immunoglobulin-like domains in its structure (2). Both T1 gene products, the soluble secreted form (T1-S) and the membrane-anchored form (T1-M) are members of the IL-1 receptor (IL-1R) family, which belongs to the immunoglobulin (Ig) superfamily (1, 2).

The IL-1R family consists of a growing number of proteins with homology to the IL-1 receptors. The cytoplasmic domains of IL-1R type I and type II are 213 and 29 amino acids long, respectively. The type II receptor is considered a decoy receptor, which binds ligands but does not trigger intracellular signaling. IL-1R type I associates with the related IL-1 receptor accessory protein (IL-1RAcP) upon ligand binding (16). Both IL-1 receptors as well as IL-1RAcP and the IL-18 receptor (17), formerly known as IL-1 receptor-related protein (IL-1Rrp) (18) share sequence similarity with T1 in their extracellular and cytoplasmic portions. Sequence comparison revealed that the ectodomains of T1 and IL-1RI are 25% identical, whereas their cytoplasmic portion share 38% identity (10).

Other proteins such as MyD88, human “randomly sequenced cDNA786” (rsc786), the human and Drosophila Toll proteins, the Drosophila protein 18-wheeler (19), and two partial Drosophila open reading frames, MstProx and STSDm2245, contain sequence homology to the IL-1RI in their signaling domain, but are associated with unrelated extracellular domains (20).

The murine and human T1 share 67.7% identity in 327 amino acid overlap (2). The genes encoding the type I and type II IL-1R and T1 are tightly linked on mouse chromosome 1 (15, 18), and on human chromosome 2 (21, 22). The highly conserved intron/exon structure of these three genes suggests a common ancestry (21).

Despite its sequence homology to the IL-1RI (10) the T1 molecule does not bind IL-1α, IL-1β or IL-1 receptor antagonist (IL-1ra) (23-25). However it has been reported by Reikerdorfer et al. that the rat homolog of T1, Fit-1S can specifically interact with IL-1β, albeit with low affinity and without exerting any signaling activities which almost excludes the possibility for IL-1β to be the physiological ligand (26).

Studies with chimeric proteins, consisting of the extracellular domain of the IL1-R and the intracellular part of T1, revealed that T1 and IL-1R trigger the same or similar signal transduction cascades such as activation of NFκ-B, the MAP kinase p38/RK, and transcription from the IL-8 promoter upon IL-1α or IL-1β stimulation (20, 23, 26).

Putative T1 ligands have been described in two reports. Gayle et al. cloned a cell surface protein from the human glioblastoma A172 cell line that is capable to bind T1, but unable
to trigger receptor activation. Moreover it displays no sequence similarity to any of the three IL-1R ligands (25). Kumar et al. have isolated two proteins of 18 kDa and 32 kDa from conditioned media of Balb/c-3T3 and human umbilical vein endothelial cells (HUVEC) that bind the extracellular domain of T1 and thereby activate MAP kinase CSBP/p38, followed by NFκB activation (23). No sequence information of these candidate T1 ligands is yet available. Another report describes the specific binding of a recombinant human T1 protein to myeloma-derived RPMI8226 and NALM-1 (CML-blastic crisis-derived pre-B) cells, indicating that B cells might express ligands for T1 assuming a possible involvement of T1 in T cell/B cell interaction (14).

### 3.2 Regulation of T1 Gene Expression

T1 synthesis in fibroblasts is associated with proliferation. As described above there is a marked increase in T1 production when quiescent cells enter the cell cycle. Growth stimulation leads to an accumulation of the T1 transcript which peaks 6-8 hours after the onset of stimulation and declines thereafter but remains at an elevated level as long as the cells are growing (7). The T1 gene can be activated through two distinct signaling pathways (Fig. 2). Growth factor- and oncoprotein mediated T1 gene expression is a delayed early event, requiring ongoing protein synthesis. The involvement of c-Fos and the Erk signal transduction pathway has been demonstrated (7, 27). In contrast, IL-1 and TNF-α mediated T1 gene induction is an immediate early event which does not require ongoing protein synthesis and which occurs via the MAP kinase p38/RK pathway (9). Consistent with this result is the finding that anisomycin which is a strong p38/RK activator also induces the T1 gene.
Figure 2. Induction and regulation of T1 gene expression.
The T1 gene can be induced by mitogens and oncogene expression through stimulation of the ERK signal transduction pathway. It is a delayed early event since Fos proteins have to be synthesized in order to stimulate the T1 gene. TNF-α, IL-1 and anisomycin however induce the T1 gene in an immediate early event through stimulation of the p38/RK pathway.

In murine fibroblasts, expression of the T1 gene is regulated by transcription factors of the AP-1 family and by E box binding proteins (7-9, 28). Within the 148 bp T1 enhancer, 3.6 kb upstream of the transcription initiation site used in fibroblasts, there is an essential TPA-responsive element (TRE), the binding site for AP proteins, and three E boxes which are important for efficient gene expression in fibroblasts (28). Overexpression of c-Fos and FosB was sufficient for T1 gene induction in these cells (7). Similarly the rat homolog of T1, fit-1, was shown to be a c-Fos-responsive gene (6). The induced expression of the Ha-ras oncogene results in the transient accumulation of c-Fos and consequently of T1-S. In contrast, sustained Ha-ras oncogene expression leads to the accumulation of Fra-1 and activation of c-Jun through JNK mediated phosphorylation.
This AP-1 complex, consisting predominantly of Fra-1 and c-Jun can repress c-Fos gene activation, preventing T1 gene activation. Thus, in ras transformed fibroblasts the T1 gene is refractory to the induction by various growth factors. However, IL-1 and TNF-α mediated T1 gene induction is not affected by the ras oncogene (27).

In mast cells, transcription initiates at an alternative exon 1, 10.5 kb further upstream than the exon 1 which is used in fibroblasts. T1 gene expression in mast cells is regulated by GATA transcription factors, in particular by GATA-1 (29). The enhancer element which is essential for fibroblast specific T1 gene activation is dispensable for expression in mast cells (13). Under normal growth conditions accumulation of the 5 kb transcript occurs. However Ca²⁺ ionophore treatment and, to a lesser extend FcεRI ligation, resulted in the rapid disappearance of the 5 kb T1 transcript and the massive accumulation of the 2.7 kb T1 mRNA followed by the secretion of T1-S (12, 13).

The different promoter usage is strictly tissue-specific, the distal and the proximal promoters are used by hematopoietic cells and fibroblasts, respectively (13).

### 3.3 Expression Pattern of T1 Transcript and T1 Protein

The two T1 transcripts differ in their expression pattern. The short 2.7 kb mRNA has been detected in the embryonic skin, retina, and bone (24), in the developing mammary gland, in Ha-ras-induced murine mammary adenocarcinomas (24), and in fibroblast cell lines (1-4). The 5 kb mRNA was detected in the major hematopoietic organs such as fetal liver, spleen and bone marrow (24), in the lung (6), as well as in several cell lines derived from macrophages, erytroid progenitors, and T cells, in a mast cell line (6, 24), in primary mast cells (13, 24, 30) as well as in Th2 effector cells (14, 31, 32).

While the T1 transcript was detected by Northern blot analysis and in situ hybridization in several of the aforementioned cells and tissues, expression of the mature membrane-anchored protein was shown by Western blot and flow cytometric analyses on mast cells (13), on mast cell progenitors in fetal blood (30), and on Th2 effector cells (14, 31, 32). Stimulation of quiescent fibroblasts with serum, growth factors, and proinflammatory cytokines or in response to oncogene expression led to the production of the soluble form of T1, as revealed by Western blot analysis (7, 9, 28).
3.4 CD4+ T cells

In adaptive immunity, specificity is mediated by lymphocytes. They can be divided into two broad classes according to their function: the B lymphocytes, representing the precursors of antibody-secreting cells, that account for the humoral immune response and the T lymphocytes that are responsible for cell-mediated immunity and for induction of B cell responses to most antigens.

Most T lymphocytes express the α/β T cell receptor (TCR), but there is also a population of T cells expressing a receptor consisting of a γ and a δ chain. The α/β TCR bearing cells are further divided into two important sublineages according to the co-receptor molecule they express: CD4 and CD8 expressing T cells. Both cell types differ greatly in the way they recognize antigens and in the types of regulatory and effector functions which they exert.

CD4+ T cells fulfill major regulatory functions such as the activation of specific B cells to produce antibodies or the increase of the microbicidal activity of macrophages. CD8+, also named cytotoxic T lymphocytes (CTLs) exert predominately direct effector functions such as destroying target cells bearing antigens recognized by the CTLs (33).

3.5 Th1/Th2 CD4+ T cells

In 1986, it was reported for the first time that CD4+ T cells could be classified into two types of effector cells, T helper 1 (Th1) and T helper 2 (Th2), based on the profile of cytokines they produce and on their related functional capabilities (34). Th1 cells were found to produce interleukin-2 (IL-2), interferon-γ (IFN-γ), and lymphotoxin (LT) and were shown to be associated with cellular effector functions such as delayed type hypersensitivity (DTH) and macrophage activation (35, 36). Th 2 cells are defined by their production of interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-9 (IL-9) and interleukin-13 (IL-13) that in turn enhance antibody production by B cells and recruitment of eosinophils to sites of immune reactions (35). In addition, there are more subsets of CD4+ T cells such as Th0 cells, that express a mixture of the two cytokine pattern (37) or Th3 cells, which produce high amounts of TGF-β but neither IL-4 nor IFN-γ (38). These subsets may consist of mixed populations of CD4+ T cells or may be intermediates in the differentiation of Th1 and Th2 cells. Although the
The Th1 and Th2 model has been widely accepted, it must be taken into account that individual T cells and clones display substantial diversity with regard to their cytokine profiles representing a continuous spectrum rather than distinct subsets in which Th1 and Th2 cells may only be two of the possible extreme phenotypes (39).

3.6 Development of Helper T cell Subsets

Upon initial encounter with the antigen, the mature naive CD4+ T helper precursor cells (Thp) produce IL-2 and subsequently differentiate into cells that secrete other cytokines. These different subsets of cells develop from the same T cell precursor (40) and their differentiation is strongly influenced by the manner and environment in which these precursor cells are stimulated. Among several different factors such as antigen concentration or co-stimulatory molecules, the most potent differentiation-inducing stimuli are the cytokines themselves. Each T cell subset produces cytokines that serve as its own autocrine growth and differentiation factor and at the same time as an inhibitor of the development pattern that leads to the opposing subset (as reviewed by Abbas et al. (41)).

IL-12, produced by activated macrophages and dendritic cells, is a dominant factor promoting Th1 differentiation. IL-12 exerts its action on Th1 development by activating the transcription factor Stat4 (42) as has been shown in mice deficient of either IL-12 (43) or Stat4 (44, 45) which showed markedly reduced Th1 responses. Another cytokine, IFN-γ, may have little direct Th1 inducing effects. However, it promotes Th1 development in several indirect ways. IFN-γ enhances IL-12 secretion by macrophages, prevents the outgrowth of Th2 cells and maintains the expression of IL-12 receptors on CD4+ T cells, rendering them more responsive to IL-12. It appears that the expression of functional receptors for IL-12 are restricted to recently activated, uncommitted CD4+ T cells and to Th1 cells and are lost upon differentiation to Th2 cells (42). Recently, a Th1-specific transcription factor, T-bet, has been isolated whose expression correlates with that of IFN-γ in Th1 cells and which directs Th1 lineage commitment from naive Thp cells by activating the developmental program that leads to Th1 differentiation and repression of Th2 development (46).

The development of Th2 cells from naive CD4+ T cells has been attributed to the exposure to IL-4 at the beginning of an immune response (40, 47, 48). It seems that T
cells themselves produce small amounts of IL-4 in response to their initial activation and that the amount of IL-4 at the site of T cell activation accumulates with increasing lymphocyte activation until it reaches the threshold level which is needed to initiate Th2 cell differentiation (49). There are several other candidates for providing IL-4 early in an immune response such as memory and possibly naive major-histocompatibility complex (MHC) class II-restricted CD4+ T cells, the NK1.1+ subset of CD4+ T cells and non-T cells such as mast cells, basophils, and eosinophils (as reviewed by O'Garra et al. (50)).

The binding of IL-4 to its receptor ultimately leads to the activation of the Stat6 signaling pathway. Deletion of the genes encoding IL-4, the IL-4 receptor or Stat6 in mice results in deficient Th2 responses (reviewed by Nelms et al. (51)). The mechanism by which Stat6 induces IL-4 production and Th2 development is unclear, but there is a consensus Stat6-binding site present in the IL-4 promoter suggesting that Stat6 may activate IL-4 transcription in response to IL-4 itself (52). Apart from Stat6, several other transcription factors have been implicated in Th2 cell differentiation. One of them, GATA-3 was found to be selectively expressed in differentiating and effector Th2 but not Th1 cells (53).

Although the above mentioned cytokines play a key role in governing the differentiation of Th1 and Th2 effector cells from naive precursors, there are at least two other factors influencing lineage commitment: dose of antigen and co-stimulation.

With few exceptions, low-antigen concentration and low-dose infection tend to induce Th1 responses, whereas high-antigen concentration and high-dose infection preferentially induce Th2 responses (54, 55).

Co-stimulatory molecules that are expressed on antigen-presenting cells (APCs) activate T-cells by interacting with their specific receptor and together with the antigen provide signals that influence both Th1 and Th2 cell development. The best-characterized costimulatory molecules are B7-1 and B7-2 that interact with CD28 on T cells (56). High levels of co-stimulation favor Th2 responses. Studies with antibodies that inhibit the activation of the CD28 pathway in a priming culture suggest that CD28 co-stimulation is important for the induction of IL-2, which in turn is essential for priming for IL-4 production (40). According to these findings it is assumed that high initial T cell activation results in increased IL-4 production promoting the IL-4-dependent autocrine pathway of Th2 differentiation. However, a more recent report describes IL-4 independent proliferation of Th2 cells. This model suggests a self-amplifying autocrine mechanism that is triggered by co-stimulation via TCR and IL-1R and leads to the
production of cell-associated IL-1α which in turn leads to IL-4 independent Th2 cell proliferation (57). The interaction of CD40 ligand (CD40L) on T cells with CD40 on APCs is another co-stimulatory pathway that regulates immune responses. Initially it was shown that the CD40/CD40L interaction was important for B cell activation and isotype switching (58), but later it has become evident, that it is also critical for the regulation of CD4+ T cell proliferation and cytokine production (59). This ligation causes up-regulation of several co-stimulatory ligands such as B7 (60) and enhances the production of IL-12 by both monocytes and dendritic cells, suggesting that this co-stimulatory signal favors Th1 differentiation (61, 62). CD4, the molecule that defines the T helper subset of T lymphocytes plays at least two roles in T cell activation. First, it binds to the TCR-MHC class II-antigen complex and thereby increases the strength of this binding, and second, it mediates signaling through the association of its cytoplasmic tail with the protein kinase p56lck (63). These functions seem to contribute to the Th2 differentiation pathway, since mice lacking CD4 were able to mount a Th1 but not a Th2 response against protein or parasite antigens (64). Overall it seems that the Th1 response is less influenced by co-stimulators than the Th2 response, and that Th1 development is strongly dependent on IL-12 production by APCs (65).

![Figure 3. T helper cell subsets.](image)

Differentiation pathways of naive CD4+ T cells towards the Th1 or Th2 subset after T cell activation and the role of the subset specific cytokines leading to the characteristic type 1 and type 2 effector functions. Th1 cells are activated by IL-12 and IFN-γ and inhibited by IL-4, whereas Th2 cells are activated by IL-4 and inhibited by IL-12 and IFN-γ.
3.7 Th1 and Th2 Effector Functions

The two distinct subsets of Th effector cells exert their specific functions primarily through the production of cytokines they produce. The major Th1 cytokine is IFN-γ that activates macrophages to enhance their microbicidal actions. IFN-γ also stimulates B-cells to produce IgG antibodies, in particular IgG2a and IgG3 in mice and IgG1 and IgG3 in humans (66). They are involved in opsonization and phagocytosis of particulate microbes. IFN-γ and TNF-β, another Th1 cell specific cytokine, recruit and activate inflammatory leukocytes that cause inflammation, such as delayed type hypersensitivity reaction (DTH) and tissue injury but at the same time provide protection against intracellular pathogens and viruses. Th1 specific cytokines, especially IL-2 and IFN-γ also promote the differentiation of CD8+ T cells that in turn participate in the elimination of intracellular microbes (as reviewed by Abbas et al. (41)).

Th2 cells exert their effector functions mainly through secretion of the key cytokines IL-4 and IL-5. IL-4 induces B cells to produce high levels of IgM and non-complement-fixing IgG isotypes such as IgG1 in mice and IgG4 in humans (66), and to switch to IgE production. This in turn leads to IgE-dependent mast cell-mediated reactions. IL-5 is crucial for activation and recruitment of eosinophils. Both cytokines, IL-4 and IL-5 are produced by the same subset of T cells. Therefore, in a typical Th2-dominated immune reaction presence of high IgE levels often coincides with high amounts of activated eosinophils as seen in allergy and helminth infection (as reviewed by O’Garra et al. and Abbas et al. (41, 50)). It has been shown that different cytokines cross-regulate each other’s development, i.e. IFN-γ stimulates Th1 development while inhibiting Th2 differentiation and IL-4 acts in the opposite way. Consequently, the emergence of one T helper subtype goes along with an inhibition of immune responses exerted by the opposing subtype. IL-4 and IL-13 antagonize the action of IFN-γ to activate macrophages and IL-10 inhibits Th1 development by suppressing IL-12 production by activated macrophages (67). Thus, several cytokines produced by Th2 cells have anti-inflammatory actions and the activation of Th2 cells often results in the inhibition of acute and chronic inflammation, including DTH reactions. This raises the possibility that Th2 cells also act as regulators of immune responses. In particular, Th2 cells may appear late in immune responses and serve to limit the injuries elicited by the Th1-mediated protective immunity. Furthermore regulation of Th1 and Th2 responses as well as prevention of the development of immunopathology are attributed to other regulatory
populations of T cells that produce TGF-β which is able to inhibit both Th1 and Th2 responses (as reviewed by O’Garra et al. and Abbas et al. (41, 50)).

![Figure 4. Effector functions of Th1 and Th2 subsets of CD4+ helper T cells.](image)

Th1 cells produce cytokines that induce cell-mediated immunity leading to eradication of intracellular pathogens such as virus and (mostly intracellular) bacteria. Th2 cells secrete cytokines that recruit eosinophils and activate B cells to proliferate and to produce IgG1 and IgE. Accordingly, Th2 cytokines are commonly found in association with strong antibody and allergic responses.

### 3.8 Expression of T1 in Th2 cells

The division of T helper lymphocytes into distinct subsets is based on their functional capabilities and the cytokine pattern they produce, although much effort has been invested in finding cell surface molecules that allow to distinguish between differentiated Th1 and Th2 cells. Several proteins have been described which are differentially expressed, but many of them are not specific for either Th1 or Th2 subsets but rather are markers for T cell activation or responses to cytokines. One of them is the chemokine
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receptor CCR3 for eotaxin, which is selectively expressed on human Th2 cells (68), but which is rapidly down regulated after T cell activation (31). Another molecule, CD30, was described as a Th2-specific marker, although it was shown to be expressed also on Th1 cells, albeit at much lower levels than on Th2 cells (69). More recently, two members of the IL-1 receptor family have been found to be selectively expressed on Th1 or Th2 cells. The IL-18 receptor was described to be a cell surface marker for Th1 cells (70) whereas the T1 protein was shown to be a stable marker for Th2 cells (14, 31, 32). Under in vitro Th2 polarizing conditions of spleen derived cells the amount of cells expressing T1 increases from approximately 5% after primary re-stimulation to about 40% after tertiary re-stimulation, while naive Th cells and Th1 effector cells fail to express T1 (71). Thus, T1 is expressed by antigen-experienced CD4+ T cells both in vitro and ex vivo which produce high amounts of IL-4, IL-5 or IL-10, but little or no IL-2 and IFN-γ. While expression of T1 correlates with Th2 cytokine production, it does not depend upon them. In IL-4 and IL-10-deficient mice the level of T1 expression is unaffected, while in IL-5-deficient mice, the T1 expression was 3-fold less compared with wild-type mice. This could indicate that regulation of IL-5 production and T1 expression may be interdependent or it could result from a reduced number of IL-4 or IL-10-producing cells in IL-5-deficient mice (32).

3.9 Function of T1 in the Th2 Response

In view of the selective expression of T1 on Th2 lymphocytes, the role of this molecule in Th2 responses has been studied using anti-T1 antibodies and a T1-Ig fusion protein. Several groups investigated the effect of anti-T1 antibodies on Th1 and Th2 cells in vitro and in vivo using different infection models in mice. Incubation of Th1 and Th2 clones with an anti-T1 antibody plus complement resulted in the specific lysis of Th2 but not Th1 cells (31). Studies with a T1-Ig fusion protein revealed that T1 signaling is important for the activation of and differentiation to Th2, but not Th1 effector cell populations. Addition of a T1-Ig fusion protein to spleen derived lymphocytes which were exposed to either neutral, Th1 or Th2 polarizing conditions, resulted in the selective inhibition of IL-4 and IL-5 cytokine production and in an augmentation of IFN-γ secretion by the cells in the neutral and Th2, but not the Th1 polarizing environment. In addition, blockade of T1 signaling also affected the cytokine production of Th2 but not Th1 effector cells in a
dose-dependent manner (71). During an immune response against *Schistosoma mansoni* eggs T1 was up-regulated on Th2 cells and this correlated with type 2 cytokine production as revealed by *ex vivo* cytokine analysis (72). Based on these *in vitro* and *ex vivo* observations one would assume that T1 is crucial for the development of a Th2 response.

Resistance and susceptibility to *Leishmania major* in inbred strains of mice is controlled by CD4+ T cell subsets. Resistant strains such as C57BL/6 mount a strong Th1 response with a strong DTH reaction, high levels of IFN-γ but little if any IL-4 or IL-5, whereas susceptible strains such as BALB/C mice predominately show a Th2-type response characterized by high antibody levels and significant IL-4, IL-5 and low IFN-γ expression (73, 74). Treatment of susceptible *L. major* infected BALB/c mice with an anti-T1 antibody resulted in the development of smaller lesions and less parasite load as compared to mice treated with a control antibody. Cytokine analysis of the draining lymph nodes revealed an enhanced IFN-γ and reduced IL-4 and IL-5 production in the anti-T1 antibody treated mice, whereas the IL-12 levels were unaffected. While the addition of T1 antibodies ameliorated the resistance to the *L. major* infection, it exacerbated the predominantly Th1-mediated disease collagen-induced arthritis (CIA) in susceptible DBA/1 mice. In both infection models the mice displayed a shift towards Th1 cell development (31). Whether these findings resulted from anti-T1 antibody mediated Th2 cell depletion remains an open question. In an adaptive transfer model whereby aeroallergen provocation of mice receiving *in vitro* polarized Th2 cells led to accumulation of eosinophils in the airways and secretion of Th2 cytokines, it was shown that pretreatment with an anti-T1 antibody or a T1IgG fusion protein reduced eosinophil infiltration by 70% and drastically reduced the Th2 cytokine secretion in the BAL fluid (32, 71). All these findings might point to a crucial function of the T1 protein in a Th2 dominated response.

### 3.10 Murine Model Systems of Infections

#### 3.10.1 *Nippostrongylus brasiliensis* infection model

Studies with nematode infected rodents have provided considerable insight into the immune mechanisms that protect against parasitic gastrointestinal nematodes. The great
majority of helminths induce strong Th2 responses accompanied by eosinophilia and high levels of IgE. The Th2 produced cytokines and the effects they elicit are crucial for the resistance to the infection.

**Life cycle of *Nippostrongylus brasiliensis***

The rat hookworm *Nippostrongylus brasiliensis* (Nb) has been adapted to the mouse for experimental purposes. In comparison to other nematode parasites, Nb has a more complex life cycle as it migrates through several different tissues during the course of infection. The infective third-stage larvae (L₃) are free-living and can penetrate the host skin (or are injected subcutaneously as has been done in this study). After 24-48 h they reach the lungs, where an inflammatory response is induced that is characterized by pulmonary eosinophilic granulomas. The larvae are coughed up, swallowed over the next 24-48 h and migrate to the small intestine where they mature into egg-laying adults by day 5-6 after inoculation. Adult worms are expelled alive, although damaged, from the gut of immunocompetent mice around day 12 post infection. The excreted eggs develop into L₁ larvae in the soil and undergo two molts to become the infectious L₃ form. Mice that were able to clear a primary infection with Nb will mount a very strong memory response upon re-challenge with infectious larvae. They will reach the gut in reduced numbers and produce very few, if any, eggs (75).

**Characteristics of the Th2-type immune response induced by *N. brasiliensis* infection**

Infection with Nb induces a strong Th2-type response characterized by peripheral blood and tissues eosinophilia, high and intermediate increase in serum IgE and IgG1, respectively and large increases in intestinal mastocytosis (76). All these immunological hallmarks of infection appear to be induced by Th2 released cytokines: eosinophilia is stimulated by IL-5, IgE and IgG1 production by IL-4 and mastocytosis by IL-4 and IL-3. In a protective immune response, the worms will be expelled from the host by day 12 post infection (77). The expulsion of worms is one of the parameters to test the efficiency of an ongoing immune response. Failure or delay in the expulsion of the parasite is indicative for the absence of a protective immune response. Despite the intensive study of the immune response to Nb infection, the exact mechanisms that are responsible for immune protection and ultimate worm expulsion are still not entirely known.
Role of CD4\(^+\) T cells

Protective immunity against Nb is dependent on CD4\(^+\) T cells. Anti-CD4 mAb, but not anti-CD8 mAb treatment prevents expulsion of adult worm and promotes parasite egg production as long as the mAb treatment is maintained. However, mice that have cleared a primary infection, are protected against re-infection, even when anti-CD4 mAb are injected at the time of the challenge infection (78). Thus, CD4\(^+\) T cells are crucial for the induction of the primary protective mechanisms but not mandatory for the memory response to re-infection.

Role of IL-4, IL-13, IgE and IgG1 production

IL-4 production by CD4\(^+\) T cells during Nb infection induces IgM\(^+\)/IgD\(^+\) precursor cells to switch to IgE and IgG1 expression, while suppressing production of IgG2a and IgG3 isotypes. Antibodies against IL-4 or IL-4R totally block IgE production but have little effect on the generation of IgG1 responses (76, 79-81). Although IL-4 is important in inducing Th precursor cells to Th2-type cells both in vivo and in vitro, it is not required for protective immunity to Nb. Mice deficient in IL-4 (82) and mice treated with anti-IL-
4 antibody (83) are able to clear the infection normally. However, it has been shown that IL-4 may contribute to effective worm expulsion since anti-CD4 mAb treated or SCID mice, which lack both T and B cells, provoke expulsion of Nb upon treatment with recombinant IL-4 (84). Mice deficient in the α chain of the IL-4 receptor (IL-4Rα) or its associated signaling molecule, Stat6, develop a chronic infection, demonstrating that another factor than IL-4 is important and signals through IL-4Rα (85). It is likely that this factor is IL-13, since IL-4 and IL-13 display overlapping activities and bind both to the type II IL-4 receptor complex, that consists of IL-4Rα and IL-13Rα1 (86). IL-13 deficient mice are unable to expel worms, indicating that IL-13 plays a major role in clearance of Nb infection (87) and that the functions of IL-4 and IL-13 are not redundant. IL-13-deficient mice also failed to generate the goblet cell hyperplasia that normally occurs with worm expulsion (87). In other nematode infections, it was described that IL-4 is involved in the induction of physiological changes that ultimately would lead to worm expulsion (75). In Nb infected mice such a function has not been ascribed to IL-4, but it seems likely that IL-13 is involved in the production of intestinal mucus which is believed to facilitate worm expulsion.

**Role of IL-5 and eosinophils**

Infection with Nb induces CD4+ T cells to produce IL-5 that in turn causes the accumulation of large numbers of eosinophils in the blood and the lungs. The induction of eosinophilia has been clearly ascribed to the action of IL-5 since antibodies against IL-5 completely suppress the increase in blood and tissue eosinophilia (88). Likewise, IL-5 deficient mice are unable to mount eosinophilia in response to parasite infection, although they produce eosinophils, albeit with 2- to 3-fold lower basal levels than wild-type mice (89). Despite the absence of eosinophilia, IL-5-deficient mice and mice treated with anti-IL-5 antibodies are able to clear the infection normally. Moreover, since eosinophilic inflammation is abrogated, they showed a markedly reduced lung damage after infection (90). In contrast, mice transgenic for IL-5 develop a persistent eosinophilia and show an increased resistance to primary Nb infection. Since only few parasites are found in the intestine and egg production is minimal it is likely that the Nb larvae are killed in the lungs or even before they reach the lungs (91). These data suggest that eosinophils exert their action by inhibiting the movement of Nb larvae during the first few hours of a primary infection, which may have profound effects on later stages of parasite development.
3.10.2 Viral models

Murine infection with lymphocytic choriomeningitis virus (LCMV) or vesicular stomatitis virus (VSV) have been widely used as model systems for studying the role of lymphocytes in viral infections.

**Lymphocytic choriomeningitis virus (LCMV) infection**

LCMV is a negative-stranded enveloped RNA virus that belongs to the family of arenaviridae. The internal nucleocapsid consists of two RNA segments and the nucleocapsid protein (NP). On the surface, the virion contains the glycoproteins G1 and G2 that are inserted in the lipid envelope. LCMV is a non-cytopathic virus that causes either acute, self-limiting infection with lasting immunity or lifelong persistence. The most common mode by which a state of viral persistence is acquired is congenital/neonatal infection, establishing a state of “immunological T cell tolerance” to the virus. Alternatively, viral variants that fail to elicit a cytotoxic T cell (CTL) response can establish persistence in immunocompetent mice. Moreover, persistently infected cells can escape immune surveillance by down-regulating the expression of the viral glycoproteins. Persistent LCMV infection is accompanied by low or undetectable levels of LCMV-specific CTL activity, little or no detectable neutralizing antibodies but virus shedding throughout the whole life span (92).

An acute LCMV infection is characterized by a vigorous T cell-mediated immune response, which also accounts for the associated pathogenesis.

Intracerebral injection of LCMV in immunocompetent mice causes a fatal choriomeningitis and the mice die within 8-10 days. In these mice antiviral CD8+ T cells cause an acute and severe inflammation that leads to death (93). As shown with CD8-deficient mice, CD4+ T cells can also mediate choriomeningitis in response to intracerebral LCMV infection, although it is less severe than the one mediated by CD8+ T cells (94).

Injection of replicating LCMV into footpads induces a CD8+ T cell-mediated swelling reaction which peaks on day 8 followed by a CD4+ T cell-mediated swelling which peaks day 11 (95). The CD8+ T cell-mediated virus-specific cytotoxic activity in mice is observed 7-9 days after infection (96) and coincides with virus clearance (as reviewed by Buchmeier et al. (97)). CD8+ T cells recognize virus-specific peptides presented by MHC class I molecules and lyse the infected cell. Perforin-dependent cytotoxicity is crucial in
the control of acute LCMV infection (98). Two epitopes on glycoprotein G1 were described to elicit neutralizing antibody production (99). Administration of anti-glycoprotein mAbs either before or after intracerebral virus challenge protected the mice from subsequent CNS disease. However, passive administration of anti-NP or anti-GP-2 mAbs had no ameliorative effect on the outcome of the infection (100). Further studies revealed that initial virus control mediated by CTL is incomplete and a CTL-independent clearance mechanism involving IFN-γ and neutralizing antibodies is mandatory for permanent virus control. Thus, neutralizing antibody-producing B cells and CD4+ T cells are required for long term virus control, while CD8+ T cells significantly contribute to virus clearance in the early phase of infection (101).

**Vesicular stomatitis virus (VSV) infection**

VSV is a cytopathic negative-stranded RNA virus. It belongs to the Rhabdoviridae and preferentially infects the nervous system. VSV infection of immunocompetent mice elicits a rapid T cell-independent neutralizing IgM response, followed by production of neutralizing IgG antibodies that is dependent on CD4+ T cell help (102). The early T cell-independent IgM response is triggered by the VSV particles themselves since they exhibit highly repetitive neutralizing determinants in a rigid, two-dimensional crystal-like form on their surface glycoproteins (VSV-G) leading to receptor cross-linking (as reviewed by Zinkernagel et al. (103)). It is known that viral infection stimulates the production of IL-12 which is expressed mainly by macrophages and it potently induces a Th1 response (104). However, it has been shown that viruses can also induce a Th1 response in the absence of IL-12 (105). Effector CD4+ T cells with a Th1 cytokine phenotype possess much greater antiviral protective capacity than their Th2 counterparts (106). CD4+ T cells also elicit direct antiviral effects via the production of cytokines such as IFN-α/β. Together with IgM they inhibit the initial virus replication in peripheral organs and the spreading of the virus to the CNS (107). While B cells play an important role early in infection, CD4+ T cells are crucial for long term survival of the mice. They provide B cell help required for immunoglobuline isotype switching and a sustained antibody response. T cell help is essential to keep the virus under control. In the absence of specific immune responses the virus will reach the CNS and the mice will die within 5-10 days post infection (p.i.) due to paralysis (108). In contrast to CD4+ T cells, class I-restricted T cells are less efficient in controlling this virus infection, although in the
absence of T helper cells, a significant antiviral potential of CD8\(^+\) T cells has been reported (109). A possible explanation why cytotoxic T cells are relatively inefficient in controlling VSV infection might be that infected cells are quite rare in the periphery making it difficult for effector T cells to encounter the target. Moreover, there is low expression of MHC class I on neurons, making it even more unlikely that T cells are activated (110).

3.10.3 *Listeria monocytogenes* model

Murine listeriosis has been used as a model for studying mammalian host defense against intracellular bacterial pathogens. *L. monocytogenes* is an enterovasive, gram-positiv, facultative intracellular pathogen of humans and other mammals. As an opportunistic pathogen it can cause systemic infection in immunocompromised individuals and in pregnant women, leading mainly to septicemia and meningitis (111). Protection against *L. monocytogenes* infection is mediated by innate (NK cells, macrophages, and neutrophils) and acquired (T cells) immunologic mechanisms. The course of infection can be separated into three stages. At each stage, the involved immune cells produce cytokines that fulfill two functions: first, they execute effector functions directed at reducing the microbial burden, and second, they perform regulatory functions which influence the subsequent course of the infection (112). Within minutes after intravenous injection of a sublethal dose of *L. monocytogenes*, more than 95% of all bacteria are removed from the blood by macrophages, especially the liver Kupffer cells and neutrophils, that are attracted to the site of listerial growth and dramatically reduce the initial listerial burden (113). Neutrophils play a key function in early anti-*Listeria* defense in the liver, as demonstrated in experiments with mice that lost their ability to kill the parasite upon either elimination of neutrophils or by preventing their accumulation at infectious foci (114). Pathogens that survive the early inactivation in the liver multiply progressively for 24 hours. *Listeria*-infected macrophages produce a variety of proinflammatory and regulatory cytokines, in particular IL-12 and TNF-\(\alpha\). This leads to the activation of NK cells, which in turn produce IFN-\(\gamma\) that enhances antimicrobial macrophage functions. This intermediate stage begins around day 1-2 and is characterized by the accumulation of NK cells and the appearance of \(\gamma\delta\) T cells. These cells provide the link between the innate and the acquired immune response. In the
following third stage, antigen-specific αβ T cells of the CD4 and CD8 phenotype are activated through IFN-γ and exert cytolytic activities. This results in effective control and eradication of the pathogen within less than ten days. Additionally, in a positive feedback loop, IFN-γ-activated macrophages secrete more IL-12, which ultimately leads to Th1 cell development. Thus, in listeriosis the immune response operates through Th1 cells with little evidence for a contribution by Th2 cells (112).

3.11 Transgenic animals

The ability to introduce foreign genes into the germline of an animal and the successful expression of the inserted gene in the organism allows to study the function of this gene in vivo. The development of the gene targeting technology, which makes it possible to generate transgenic animals, has led to new insights into mechanisms of development and developmental gene regulation, into actions of oncogenes, into the complex cell interactions within the immune system, and many other biological phenomena. Non-vertebrate model organisms have provided enormous information about deciphering gene functions, but are inadequate for modeling many complex mammalian physiological traits such as control of blood glucose, cardiac function, behavior and many others. For such studies mice are the animals of choice since they are physiologically and genetically similar to humans and most human genes appear to have a related mouse version, making it possible to gain insight into human diseases using mice which have been genetically manipulated such that they mimic the human disease. Most of these studies have relied on two important characteristics of transgenic mice: the tissue-specific expression of foreign genes and the selective disruption of genes of interest by homologous recombination.

In 1976 Jaenisch and colleagues accomplished the stable introduction of a single copy of proviral Moloney murine leukemia virus (MoMLV) DNA into the mouse genome, with transmission of the inserted genome as a Mendelian trait, resulting in the generation of the first transgenic mouse strain. Infection of mouse embryos with retroviruses carrying foreign genes constitutes one method for insertion of DNA into the germ line. Another more commonly used technique is the direct microinjection of recombinant DNA into the pronucleus of the fertilized egg. In order to introduce distinct mutations in the genome, efforts were made to obtain cell lines that could be manipulated in culture prior to
insertion into animals, but which maintain their pluripotency including the ability to colonize the germline. In 1981 Kaufman and Martin isolated such cell lines from early mouse embryos. These embryonic stem cells (ES cells) can maintain karyotypic normality for extended time in culture, serve as recipients for gene transfer and are able to contribute to the germline when injected into host blastocysts. Two of the aforementioned methods that were used in this work for generating transgenic animals are depicted in Fig. 6.

**Generation of transgenic mice**

- **Gene of interest**
- **Microinjection of foreign DNA into pronucleus**
- **Implantation of eggs into pseudopregnant mouse**
- **Offspring**
- **DNA analysis**

**Generation of gene knock-out mice**

- **Culture and manipulation of embryonic stem cells**
- **Blastocyst injection and embryo transfer**
- **Chimeric mouse A+B**
- **Wild type mouse A/A**
- **Germline transmission**

**Figure 6.** Outline of the procedures to obtain mice that over-express a specific gene or that carry a targeted mutation in a gene of interest.

**Microinjection of DNA into the pronucleus**

To obtain expression of a foreign gene in a specific tissue, one-celled fertilized eggs are retrieved from females and the cloned DNA is directly microinjected into the male pronucleus. The surviving embryos are reimplanted into pseudopregnant females and the resulting offspring can be tested by polymerase chain reaction (PCR) or Southern blot analysis for the successful integration of the foreign DNA into the genome. The tissue in which the injected gene is expressed can be determined by putting the gene of interest under the transcriptional control of a tissue-specific promoter. Thus, the recombinant
DNA to be injected must harbor, apart from the particular gene, sequence information determining tissue specificity including cis-acting elements for initiation of transcription.

**Embryonic stem cells**

Embryonic stem cells are derived from the inner cell mass of blastocysts and can be kept in culture for prolonged periods of time without losing their pluripotent state nor changing their normal karyotype. Genes can be introduced into ES cells by electroporation and the cell clones are selected for the presence of foreign DNA. The targeting vector contains two regions of homology to the target gene, located on either site of a positive selection marker (i.e. neomycin resistance gene), which can be used to select for stably transfected ES cells. A cross-over event on both sites leads to the deletion of the intervening sequences. The ES cells whose DNA has undergone homologous recombination with the targeting vector resulting in the replacement of one endogenous allele can be identified by PCR. They are microinjected into normal blastocysts, which are subsequently reimplanted into the uteri of pseudopregnant foster mothers. If the mutant ES cells have contributed to an offspring, chimerical animals develop and these chimeras can usually be recognized by the mosaic coat color pattern. If the microinjected ES cells derived from a brown mouse and are injected into a blastocyst of a black mouse, the chimeric animals exhibit a mixture of black and brown sections in the fur and the contribution of each color is indicative of the extent of the chimerism. Backcrossing of the chimera to a black mouse usually results in some brown mice indicating that the ES cells have participated in the germline formation in the chimeras (as reviewed by Gordon *et al.* (115)).

**3.12 Introduction to this work**

In order to elucidate the functional role of the Tl protein in an organism we set out to generate two different kinds of transgenic mice: A Tl-deficient mouse that lacks part of the Tl gene and a Tl-Fc transgenic mouse that over-expresses a hybrid protein consisting of the soluble form of Tl fused to the Fc domain of a human IgG. The Tl-Fc fusion protein is constitutively produced in the liver due to a liver specific promoter, which directs transgene expression and is secreted into the bloodstream. We assume that the putative Tl ligand is scavenged by binding to this excess of soluble receptor and is
therefore prevented from interacting with its cognate receptor at the biological relevant target site.

Recent reports describe the localization of the T1 protein on Th2 cells, suggesting that the T1 molecule might be involved in the Th2 immune response. Therefore, we used an infection model system that is known to elicit a Th2 dominated immune reaction and studied the characteristic parameters in wild-type as well as in our transgenic and knock-out mice. Infection of mice with the parasitic nematode *Nippostrongylus brasiliensis* is a widely used, well-defined model for the Th2 immune response. Furthermore we examined a possible function of T1 in the immune response against viral and bacterial infections.
4. Results

4.1 Generation of T1-deficient and T1-Fc transgenic mice

4.1.1 T1-deficient mice

In an attempt to elucidate the biological function of the T1 protein, we generated mice that lack part of the T1 gene. In order to inactivate the endogenous T1 gene, a targeting construct was designed that upon integration by homologous recombination at a specific site of the T1 gene should lead to T1 gene inactivation. The construct was designed such that its integration at the homologous sites places the LacZ gene under the control of the fibroblast specific T1 promoter and eliminate part of the T1 coding region.

![Targeting construct pKO4.](image)

Upon homologous recombination of the construct with the chromosomal DNA the LacZ reporter gene is placed under the control of the T1 promoter and 4.5 kb of the T1 gene (exon 3-6) is deleted. The neomycin gene used for positive selection with G418 is under the control of the phosphoglycerate kinase (PGK) promoter. The filled black boxes and bars indicate the T1 sequences used for homologous recombination.

The LacZ gene was included in the construct for studies of tissue specific T1 gene expression in the mouse embryo. Whole embryos or sections thereof can be stained to detect β-galactosidase activity. For the targeting construct isogenic DNA was used to improve the targeting efficiency. Gene targeting was performed using a replacement vector containing two regions of the T1 gene, a 5' ‘short arm’ of 1 kb length and a 3’ ‘long arm’ of 6.2 kb flanking the LacZ and neomycin gene. Homologous recombination
within these two regions with the T1 gene results in the deletion of the region in-between (part of exon 2, including the translation start codon, and exons 3-6). This construct was linearized and electroporated into embryonic stem cells (ES cells), which derived from the inner cell mass of a blastocyst stage embryo. ES cells were grown on a fibroblast feeder layer that provided factors that maintained the ES cells in an undifferentiated state and enhanced proliferation. After electroporation of the targeting construct, ES cells were grown in selection culture medium to allow those cells to survive that contained the neomycin gene, thereby selecting for ES cells that harbor the targeting construct. To test whether homologous recombination had taken place at the T1 locus, PCR analyses were performed using approximately half of the cells that were contained in each of the single isolated cell clones. The rest of the cells were plated on 48-well plates. For the first round of PCR screening, the cells of four clones were pooled in order to reduce the number of PCR analyses. Prior to the analyses of pKO4-transfected cells we established PCR conditions to detect the indicative PCR product in as little as 100 cells, using an ES cell clone that was stably transfected with the test-targeting construct pKO3. This construct is essentially the same as pKO4 but contains a larger 5' T1 region that covers also the sequence of the upstream PCR primer. Therefore, the PCR product, which is indicative for homologous recombination of chromosomal DNA with the pKO4 construct, is also detectable in cell lines with randomly integrated pKO3. With the linearized targeting construct pKO4 we performed two independent electroporations and isolated 482 clones. Subsequent PCR analysis revealed that three pools, each representing the DNA of four cell clones, gave rise to a band of the expected size of 1.2 kb (Fig. 8A). Reanalysis of single cell clones revealed that clones 2a and 9b contained a disrupted T1 allele (Fig. 8B). From the third pool only three clones could be propagated, and they were all negative in PCR analysis. The two positive cell clones were expanded and stored in aliquots at −70 °C until injection. A small number of the ES cells were kept in culture to test the karyotype of the cells. For this purpose the chromosomes were arrested in their metaphase and the DNA was stained with DAPI. The cell nuclei were mechanically disrupted and the chromosomes observed under a fluorescence microscope. The chromosomal composition of at least ten cells was analyzed. Most counts revealed 40 chromosomes with a few exceptions where only 38 chromosomes were counted.
Figure 8. PCR analyses to test homologous recombination of the targeting vector with the chromosomal DNA.

10 μl of PCR product were separated on a 1% agarose gel that was subsequently stained with SYBR gold.

A) Lane 1, 17: molecular weight marker, lane 2: negative control without added cells, lane 3: negative control with non-transfected ES cells, lane 4: 10^3 pKO3-transfected ES cells, lane 5: 10^3 pKO3-transfected ES cells, lanes 6-16, 18-32: pools of four ES cell clones. Pool 2 (lane 15) and 9 (lane 23) show a band with the expected size of 1.2 kb (arrowheads).

B) Re-PCR with cells from expanded clones 2a and 9b. Lane 1: molecular weight marker, lane 2, 3: negative control without added cells, lane 4: reamplification of PCR sample of clone 9b, lane 5: non-transfected ES cells, lane 6: clone 2a, lane 7: clone 9b.

Clone 9b was injected into 120 blastocysts which were subsequently implanted into ten pseudopregnant females. Four of these mice gave birth to a total of 15 offspring, of which four males were highly chimeric (90-95%). The degree of chimerism is estimated by the fraction of brown patches in the otherwise black fur coat. The brown spots in the coat indicate that the manipulated ES cells have contributed to some of the tissues of the mouse. Two highly chimeric males were bread to C57BL/6 females and the offspring monitored for their fur color. Since the agouti color is dominant, the manipulated DNA must have been transmitted through the germline in animals with agouti coat color. From the first backcross of two highly chimeric males to C57BL/6 females 17 F1-animals were obtained, all of them with brown coat color. PCR and Southern blot analysis revealed that 11 animals contained a disrupted T1 allele. To obtain homozygous T1-deficient mice, heterozygous F1-mice were inbred yielding wild-type, heterozygous and T1-null mice in a normal Mendelian ratio.
Absence of T1 protein in vivo

While PCR and Southern blot analyses (page 55, Fig. 18) revealed a disruption of the T1 gene in T1-deficient mice, the absence of the T1 protein in vivo had to be proven as well. Therefore we isolated bone marrow cells of T1-deficient mice by flushing femurs and tibias. These cells were cultured in the presence of IL-3 to enrich for mast cells. After about four weeks in culture the cells displayed a typical IgE⁺ and c-kit⁺ phenotype as revealed by FACS analysis (data not shown). These cells were analyzed for the absence of T1 expression by immunoblotting with a rabbit polyclonal antiserum (Fig. 9A) and by flow cytometry using an anti-T1 mAb (Fig. 9B).

![Figure 9. Absence of T1 protein in mast cells of T1-deficient mice.](image)

A) Bone marrow derived mast cells of T1-deficient and wild-type mice were lysed by boiling in reducing Laemmli sample buffer. 15 and 30 µg protein were subjected to SDS-PAGE in an 8% SDS-polyacrylamid gel and electroblotted onto a PVDF membrane. After blocking unspecific binding sites with 5% milk in PBS, the membrane was incubated with a rabbit anti-T1 polyclonal Ab. Binding of the antibody was revealed by incubation with an HRP-conjugated goat anti-rabbit Ab. No binding was seen in mast cell lysates of T1-deficient mice (lane 2 and 3), whereas a band was present in mast cell lysates deriving from wild-type mice (lane 4 and 5). As a positive control, 2µg of recombinant T1-Fc protein was loaded in lane 1. B) Mast cells of T1-deficient and wild-type mice were double stained with FITC-conjugated anti-T1 (DJ8) and PE-conjugated anti-c-kit mAbs. T1 expression was analyzed by two-color dot plot (upper panels) or by histogram analysis (lower panel). The isotype control is shown by the dotted line.
4.1.2 Immunological profile of T1-deficient mouse

Apart from mast cells, Th2 cells also show expression of T1 (14, 31). So far, these two cell types have been the only cells of the immune system for which T1 expression has been described. Naive T cells fail to display the T1 molecule on their surface, but upon antigenic stimulation a small fraction of the CD4+ T cells coexpresses T1 and type 2 cytokines indicating that these cells are committed to the Th2 phenotype (32, 71). In the absence of T1 this T cell population might be affected in its development or cell number. To examine whether the absence of the T1 protein has an influence on the lymphocyte composition, splenocytes, thymocytes and bone marrow cells were isolated from T1-deficient mice and subjected to FACS analysis. In the thymus immature T cells or thymocytes undergo the gene rearrangement that produces the T cell receptor, and the positive and negative selection that shapes the mature receptor repertoire. The most important cell-surface molecules to identify thymocyte subpopulations are CD4, CD8 and the T cell receptor. These molecules allow the distinction of thymocyte populations at different stages of maturation. While the earliest cell population in the thymus does not express any of these molecules, thymocytes at a later stage of development express both CD4 and CD8 on their surface. Most of these cells die in the thymus as a consequence of failed positive selection, but those cells whose receptors bind self MHC molecules lose expression of either CD4 or CD8, depending on MHC restriction. The resulting mature single-positive T cell leaves the thymus to enter the peripheral circulation.

A large proportion of cells coexpressing CD4 and CD8 as well as a CD4 and CD8 positive subpopulation were present in the thymus. While the number of CD4+ T cells varied between 6 and 7% and of CD8+ T cells between 2 and 3.5%, the majority of thymocytes were CD4 and CD8 positive in both T1-deficient and wild-type mice (Fig. 10). The amount of CD4+ or CD8+ T cells in the spleen of T1-deficient mice was also compared with splenocytes of wild-type mice. The CD4+ T cell population varied between 14 and 18% and the CD8+ T cell population between 5 and 10% in both T1-deficient and wild-type mice (Fig. 10). To extend our analysis for B cells we isolated bone marrow cells and stained them with antibodies against CD45R which is an early marker of B cell development, IgM that appears later in B cell maturation, and T1. The proportion of immature CD45R expressing B cells varied between 11 and 20% and of pre-B cells positive for CD45R and IgM between 5 and 8% irrespective of their genotype. Taken together, it appears that neither the T cell development nor the B cell maturation is affected by the absence of the T1 protein.
In addition, a small T1 positive population was detected in bone marrow cells of wild-type mice (data not shown). These cells probably represent immature mast cell precursor cells as described by Moritz et al. (30).

Figure 10. Lymphocyte composition of T1-deficient mice.
Splenocytes, thymocytes and bone marrow cells were isolated from 9-week-old T1-deficient and wild-type mice. Splenocytes and thymocytes were stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 mAb. Bone marrow cells were stained with PE-labeled anti-B220 and FITC-labeled anti-IgM mAbs. The proportion of T and B cells in the different organs were analyzed by two-color dot plot and is indicated in the quadrants as percentage of total counted cells. Two mice per group were analyzed and revealed similar values.
4.1.3 Expression pattern of Tl in embryos

Previously Rössler et al. had investigated the expression pattern of the soluble and the membrane bound form of Tl in embryos and adult mouse tissues by RNA analysis and in situ hybridization. Northern blot analysis of entire embryos revealed that the 5 kb mRNA was faintly detectable from days 10 to 14 of gestation and increased during later embryogenesis. In situ hybridization showed high signal intensity for the 5 kb Tl mRNA in single dispersed cells of the liver at embryonic day 14 to 19. No signal was observed in the embryonic spleen. Single high Tl mRNA expressing cells were also detected in bone marrow from embryonic day 19 to adult mice. The 2.7 kb mRNA was not detected by Northern blot analysis, but RT-PCR analysis demonstrated expression from day 10 of gestation until partuition. Using in situ hybridization the 2.7 kb Tl transcript was found to be predominately expressed in nonhemopoietic embryonic tissues such as the developing eye, bone and skin. In adult mouse tissues both transcripts were observed in moderate amounts in bone marrow and spleen and in low amounts in thymus (24).

Homologous recombination of the pKO4 targeting construct with chromosomal DNA placed the LacZ gene under control of the Tl promoter. This enabled us to follow the site of Tl expression by detecting the expression of β-galactosidase with an anti-β-galactosidase antibody. To control whether the LacZ gene was indeed expressed in the expected tissue we prepared cytopsins of mast cells from wild-type and knock-out mice and stained them for β-gal activity (Fig. 11). As expected, strong β-gal activity could be detected only in mast cells derived from knock-out mice. To search for β-gal expression during embryonic development we prepared cryo-sections of Tl-deficient and wild-type embryos at days 10, 12, 14, 16, and 18 of embryonic development and stained them with an anti-β-galactosidase antibody. As a positive control, cytopsins of bone marrow derived mast cells from Tl-deficient mice were subjected to the same staining procedure. Surprisingly, and in contrast to the data of Rössler et al. there was no expression of β-galactosidase visible. However, when whole embryos were stained with the β-galactosidase substrate Bluo-Gal we observed blue staining of the skin. Upon microscopic inspection of the stained skin we could not identify single β-galactosidase expressing cells. We rather observed diffuse staining of the whole epidermis (Fig. 11C, D).
Figure 11. Lac Z expression is detectable in T1-deficient mast cells and embryonic skin.
Cytospins were prepared from in vitro cultured bone marrow-derived mast cells of T1-deficient and wild-type mice. Cells were fixed with aceton, stained with a polyclonal rabbit anti-β-galactosidase antibody, followed by an AP-labeled goat anti-rabbit and an AP-labeled donkey anti-goat antibody to enhance the signal. The reaction was visualized with naphtol AS-BI phosphate and new fuchsins. Positively stained cells exhibit a bright red color. Cytospins of T1-deficient (A) and wild-type (B) mast cells. T1-deficient (C) and wild-type (D) embryos at day 16 of embryonic development were fixed in 4% paraformaldehyde for 90 min. and stained with the chromogenic substrate Bluo-gal (1 mg/ml) in staining buffer.
4.1.4 T1-Fc transgenic mice

Another approach to clarify the function of the T1 protein was to generate a transgenic mouse that overexpresses the soluble form of the T1 molecule in the blood. The presence of high levels of soluble T1 in the blood stream might scavenge the putative T1 ligand and thereby inhibit it from interacting with its cognate receptor. Two different DNA constructs were made; one contains the sequence of the soluble part of the T1 protein while the other encodes the extracellular domain of T1 fused to the Fc portion of human IgGγ1. In both constructs, the T1 gene is placed under the regulation of the liver-specific α-1 antitrypsin (AAT) promoter (Fig. 12).

![Diagram of AAT-T1 and AAT-T1-Fc constructs]

**Figure 12. AAT-T1 and AAT-T1-Fc construct.**
Constructs used to generate T1-transgenic (A) and T1-Fc transgenic (B) mice. sT1 designates the extracellular region of T1 and is placed under control of the liver-specific α-1 antitrypsin promoter. The insertion of a lox sequence was used for cre-mediated recombination to excise multiple adjacent copies of the inserted transgene. The arrowheads indicate the position of the primers used for PCR and the black bars represent the probes used for Southern blot hybridization to detect transgene insertion into the genome.

Before generating transgenic mice, the transgene constructs were tested for their functionality in the human hepatoblastoma cell line HepG2. The cells were transfected with the transgene constructs using the SuperFect Transfection Kit, as described in Materials and Methods. 24 and 48 h after transfection, the cell supernatants were harvested, concentrated and tested for the presence of T1 and T1-Fc protein by immunoprecipitation (Fig. 13).
Figure 13. T1 and T1-Fc production in transfected HepG2 cells.

HepG2 cells were transfected with the constructs AAT-T1 or AAT-T1IgG. After 24 and 48 h the cell supernatants were collected and concentrated. The supernatant of AAT-T1 transfected cells was incubated with protein-G-Sepharose beads that had previously been coated with the anti-T1 mAb DJ8, whereas the supernatant of AAT-T1IgG transfected cells was incubated with untreated protein-G-Sepharose beads. After immunoprecipitation, bound proteins were eluted from the beads by boiling in reducing Laemmli sample buffer and resolved on a 10% SDS-polyacrylamide gel followed by transfer onto a PVDF membrane. Immunoprecipitated T1 (lane 3) and T1-Fc (lane 4) protein was detected with a rabbit antiserum that was raised against a bacterially expressed T1 protein. The supernatant of serum-starved NIH 3T3 fibroblasts that were induced to proliferate by the addition of 10% FCS served as positive control (lane 2). Serum stimulation leads to strongly increased secretion of the soluble form of T1. The supernatants of serum starved (lane 1) and serum stimulated (lane 2) NIH 3T3 cells were treated as described for the AAT-T1 transfected cells. As a negative control, we used cells that were transfected with a plasmid in which the AAT-T1IgG was cloned in the anti-sense orientation (lane 5).

These experiments revealed that the AAT-T1 as well as the AAT-T1IgG transgene could direct the production of T1 or T1-Fc protein when transfected into a human liver cell line. Next, we excised the transgenes AAT-T1 and AAT-T1IgG from the plasmids, and purified and microinjected the DNA fragments into the pronucleus of fertilized oocytes, derived from the hybrid strain B6C3F1. For the AAT-T1 construct we obtained 37 offspring; PCR screening revealed that seven of them carried a transgene insertion in their genome. For the AAT-T1IgG construct, we obtained six out of 25 mice that harbored the transgene. All founder-mice were backcrossed with C57BL/6 mice and the F1 generation was tested for transgene insertion by PCR. To reconfirm the PCR results and to estimate the approximate copy number of inserted transgenes Southern blot analyses were performed. Digestion of genomic DNA with HindIII yields a 3.2 and a 2.2
kb band for Tl and Tl-Fc transgene insertion, respectively. The signal intensities of both bands are very variable, indicating that different numbers of transgene copies had been integrated into the genome giving rise to different transgenic lines. In contrast, the endogenous Tl bands of 5.9 and 7 kb reveal similar signal intensities (Fig. 14).

Figure 14. Southern blot analyses of Tl and Tl-Fc transgenic mice.
DNA from tails of Tl (A) and Tl-Fc (B) transgenic mice was extracted and digested with HindIII. 5 µg of digested chromosomal DNA was separated on a 0.9% TAE gel and transferred onto a nitrocellulose membrane. A) The blot containing DNA of Tl transgenic mice was hybridized with a DIG-labeled 2.0 kb HindIII-EcoR1 fragment (Fig. 12) of the AAT-Tl transgene. Endogenous Tl sequences give rise to a 5.9 kb and a 7 kb band (filled arrowheads) whereas the band arising from the transgene is 3.2 kb in size (open arrowheads). The different intensities of the hybridization signals reflect the different copy number of integrated transgenes. B) The DNAs of the Tl-Fc transgenic mice were probed with a DIG-labeled 2.2 kb HindIII fragment of the AAT-TlIgG construct (Fig. 12), which recognizes a 2.2 kb band that is indicative of transgene integration into the genome. DNA from non-transgenic animals was loaded in the first lanes next to the molecular size marker lane in A) and B).

Mice that were shown to harbor the Tl or Tl-Fc transgene were tested for the expression of the Tl and Tl-Fc protein. Since the transgenes are expressed under the control of a liver-specific promoter, liver tissue was removed, homogenized and the proteins were separated by SDS-PAGE. After transfer onto a PVDF membrane the Tl and Tl-Fc protein were incubated with an anti-Tl rabbit antiserum followed by horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG antibody. Among all 13 transgenic lines, there was a single one, designated TgN(AATsT1Fc)423Zbz, that accumulated detectable levels of the Tl-Fc fusion protein in the liver (page 58, Fig. 19). To verify the presence of soluble T1-Fc protein in the blood of this transgenic line, serum was tested by ELISA using an anti-Tl mAb. The protein levels were high, ranging from 130-210 µg/ml in hemizygous and around 400 µg/ml in homozygous mice (page 58, Table 1). To further analyse the expression of the Tl or Tl-Fc transgenes in the other transgenic lines,
Northern blots were prepared with RNA isolated from the liver of these animals. However, no mRNA derived from the transgenes was detectable (data not shown). Since insertion of multiple adjacent copies of the transgene might inhibit transgene expression, fertilized oocytes of a non-expressing T1 and T1-Fc line were treated with Cre-recombinase to eliminate all but one copy of the transgene within a concatamer. Offspring were tested for transgene insertion in the genome and the production of the T1-Fc protein in the liver of the transgenic animals was analyzed as well. Although the copy number of the transgene was reduced as revealed by Southern blot analysis, no T1 or T1-Fc protein was detectable in the liver (data not shown). Thus, the line TgN(AATsT1Fc)423Zbz remained the only transgenic line that expresses the T1-Fc protein and hence was used for further analyses. Transgenic animals were continuously backcrossed to C57BL/6 mice and the experiments were performed with hemizygous transgenic animals and their non-transgenic littermates deriving from the backcrosses.
4.2 *In vitro* Studies with Th1 and Th2 cells

Yanagisawa *et al.* (14) found by RT-PCR that the T1 gene was expressed in hematopoietic cell lines, specifically in a Th2 cell line. The Th2-specific expression of T1 was later verified by Xu *et al.* (31) using differential display PCR comparing RNA of a panel of Th1 and Th2 clones. Moreover, antibodies against a peptide derived from the membrane form of T1 strongly labeled the surface of cloned Th2s but not Th1s.

**Polarization and functional analysis of Th1 and Th2 cells *in vitro***

To reconfirm the selective expression of T1 on Th2 but not on Th1 cells, we isolated CD4⁺ T cells from tg7 transgenic mice (116), that express a MHC class II (I-A<sup>b</sup>)-restricted TCR specific for a peptide (p8) derived from the glycoprotein of VSV on 50% of CD4⁺ T cells. CD4⁺ T cells were obtained by magnetic cell sorting (MACS) with anti-CD4 microbeads and stimulated *in vitro* with p8 bearing antigen-presenting cells (APCs). To induce the differentiation of Th1 cells, recombinant murine IL-2 was added and Th2 development was induced with recombinant murine IL-4, polyclonal sheep anti-IFN-γ antibodies and IL-2 that were added to the culture medium. The cells were subjected to three rounds of antigenic stimulation. Each time an aliquot of cells was taken for flow cytometric analysis. Intracellular cytokine staining showed that polarized Th1 cells secrete high amounts of IFN-γ and no IL-4, whereas Th2 effectors produced high levels of IL-4 and little IFN-γ. Some heterogeneity existed within these effector populations in that the Th1 effector cell population contained cells producing IFN-γ and/or TNF-α, whereas the Th2 effector cell population contained cells producing IL-4 and/or IL-10. Surprisingly, Th2 effectors produced also a substantial amount of TNF-α and some IFN-γ (Fig. 15). As for IFN-γ, there are studies describing the coexpression of IL-4 and IFN-γ in TCR-transgenic cells *in vitro* (117). The same might also be the case for TNF-α.
Figure 15. Characterization of polarized Th1 and Th2 cells.
Intracellular cytokine staining of Th1 and Th2 effector cells 10 days after second antigenic stimulation. Aliquots of 5 x10^7 cells were stimulated in vitro with PMA and ionomycin for 4h and stained with PE-conjugated anti-IL-4, anti-IFN-γ, FITC-conjugated anti-IL-10, anti-TNF-α and anti-IFN-γ. The gate was set on CD4+ T cells.

In the course of Th1 and Th2 polarization, the cells were analyzed for T1 surface expression. T1 was shown to be exclusively expressed on polarized Th2 cells and not on polarized Th1 or naive cells. The percentage of T1-positive cells increased after each round of antigenic stimulation, but seems to be restricted to a subset of Th2 cells (Fig. 16). Intracellular FACS analysis revealed that the majority of T1-positive cells produced IL-4 and/or IL-5 indicating that these cells are indeed of the Th2-type (data not shown).
Figure 16. Expression of T1 on Th1/Th2 cells in vitro.

Aliquots of 5 x 10^5 spleen cells under Th1- and Th2-polarizing conditions were stained with TriColor-conjugated anti-CD4 mAb and FITC-conjugated anti-T1 (DJ8) mAb. The overlay of the histograms revealed T1 expression in the Th2 but not the Th1 subset of CD4^+ T cells. Cells were stained before the second (A) and third (B) antigenic stimulation and nine days after the third stimulation (C). Dot blot analysis showing the appearance of T1 expression on Th2 cells after antigenic stimulation are presented in (D).

Since we and others (14, 31, 32) have shown that T1 is only expressed on Th2 and not Th1 cells, we addressed the issue whether T1 plays a critical role as a signaling molecule required for Th2 proliferation and effector functions by performing a number of in vitro studies. In a first experiment we tried to inhibit T1 activation and examined the effect of this treatment on T cell proliferation. Naive CD4^+ T cells from spleen of tg7 transgenic mice (116) were isolated and subjected to three rounds of antigenic stimulation. Nine days after the third round of in vitro stimulation, polarized Th1 and Th2 effector populations were activated with immobilized anti-CD3 mAb in the presence of different concentrations of T1-Fc or anti-T1 mAb DJ8 (20 μg/ml starting concentration with 3-fold dilution steps). The mAb DJ8 used for FACS staining as well the recombinant T1-Fc fusion protein were produced in our laboratory (12). We assume that binding of anti-T1 mAb DJ8 to T1 prevents the interaction of the receptor with its ligand whereas T1-Fc scavenges the putative T1 ligand thereby preventing T1 activation. Nevertheless, Th1 and Th2 effector cells mounted similar proliferative responses as determined by [³H]thymidine incorporation in the presence of these two reagents (data not shown).
A possible explanation for the inability of the anti-T1 mAb to influence the proliferative response of Th2 cells might be the random distribution of the T1 antibody in the culture medium. It is known from the costimulatory molecule CTLA4, that inhibitory effects on T cell proliferation only occurred when CTLA4 molecules were ligated in proximity to signaling TCR complexes (118). Therefore, we used microbeads co-coated with anti-CD3 mAb and different concentrations of anti-T1 mAb or control anti-IgG1 antibody. Th1 and Th2 effector populations were incubated with these antibody-coated beads in the presence of an anti-CD28 mAb that activates the costimulatory signaling cascade. The proliferative capacity of the cells was determined after 24, 48 and 60 h incubation and was comparable in all experiments irrespective of the added antibodies and the concentrations used. In parallel, cells were incubated under the same conditions and after 48 h the supernatant was collected and analyzed for IL-4, IL-5 and IFN-γ cytokine production by ELISA. Th1 cells produced little amount of IL-4 and high levels of IFN-γ, whereas Th2 cells secreted little IFN-γ and high amounts of IL-4 and IL-5. Treatment with anti-T1 mAb had no effect on the IL-4 and IFN-γ cytokine secretion of Th1 or Th2 effector cells as compared to control antibody-treatment. The IL-5 cytokine production was slightly but consistently enhanced in anti-T1 mAb-treated Th2 cells. However, the IL-5 production of Th1 cells was almost as high as that of Th2 cells and a similar elevated level of IL-5 production was observed in these Th2 cells upon anti-T1 mAb treatment. These experiments are difficult to interpret. Neither do we know whether DJ8 inactivates or even activates T1 or whether it has no effect. Thus, the obtained results could mean that DJ8 was not active in these experiments or that it was active but whatever activity (stimulation or inhibition of T1) did not affect the tested parameters. To extend these studies, Th1 and Th2 effector cells should be activated as in the former experiment but in the presence of T1-Fc fusion protein instead of DJ8 to test whether T1-Fc can scavenge all of the ligand, or whether binding of T1-Fc to the putative ligand prevents receptor activation that in turn could affect the cytokine production of Th1 and Th2 cells. Similar studies performed by Coyle et al. showed a significant reduction of Th2 cytokine production and an increase in IFN-γ secretion of in vitro polarized Th2 cells in the presence of T1-Ig fusion protein (71).

However, since we do not know what biochemical signals result from T1 activation we cannot test the effects of these reagents on T1 stimulation. To more systematically approach this question we need to know what ligand binds to T1 or whether there is a T1 ligand at all. With the putative T1 ligand in hands one could determine what biological
read out could be used for T1 receptor activation and then one could search for conditions under which T1 signaling is prevented or activated. But why should one do that? The most conclusive results should be obtained with the T1-deficient mouse.
Figure 17. Cytokine production of Th1 and Th2 effector cells is not altered after anti-T1 mAb treatment.

Aliquots of $10^5$ in vitro polarized Th1 and Th2 cells were incubated with $10^5$ microbeads that had previously been coated with anti-CD3 mAb (10 μg/ml) and different concentrations of anti-T1 or control antibody (5, 10, 15 and 20 μg/ml) in IMDM containing anti-CD28 mAb for 48h. Supernatants were collected and tested for cytokine production by ELISA. IL-4, IL-5 and IFN-γ production of Th1 (A, C, E) and Th2 (B, D, F) cells. Closed and open bars indicate Th1 and Th2 cells incubated with anti-T1 mAb and control antibody, respectively. The ELISA was performed in duplicate and each value is given by a separate bar. The scale in each graph is adjusted to the highest amount of cytokine production.
4.3 T1-deficient and T1-Fc transgenic mice develop a normal protective Th2-type immune response following infection with *Nippostrongylus brasiliensis*.

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Running title: T1-deficient and T1-Fc transgenic mice can generate a normal Th2 immune response.

Key words: T1, Th2 response, *Nippostrongylus brasiliensis*, gene targeting, transgenic.

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Abbreviations: **BAL**: Bronchoalveolar lavage, **N. brasiliensis**: *Nippostrongylus brasiliensis*, **T1-Fc**: Extracellular domain of T1, fused to the Fc portion of human IgGγ1.

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Summary

The interleukin-1 receptor-related protein T1 is expressed on the surface of Th2, but not Th1 cells. Studies with anti-T1 monoclonal antibodies have suggested that T1 is critical for development of normal Th2-type responses. In order to elucidate the in vivo role of T1, we generated both T1-deficient mice, and a T1-transgenic strain which secretes soluble T1-Fc fusion protein into the serum. These were analyzed for the Th2 immune response induced by infection with the parasitic nematode *Nippostrongylus brasiliensis*. Although Th2 cytokine production by lymph node cells was similar in all groups of *N. brasiliensis*-infected mice, a decrease in IL-5 production by lung lymphocytes was detected in both T1-deficient and T1-Fc-transgenic mice compared to control littermates. This difference in IL-5 production did not influence blood eosinophilia, but recruitment of eosinophils into lung tissue, especially in T1-Fc transgenic mice was slightly decreased. However, induction of all other immune parameters was normal and both T1-deficient and T1-Fc transgenic mice were able to clear the parasite infection within 12 days with similar kinetics to control mice. Therefore, in contrast to previous suggestions, we conclude that the T1 protein is not obligatory for normal development of Th2 immune responses.
Introduction

CD4+ T-helper lymphocytes can be divided into two distinct functional subsets, Th1 and Th2, based on the profile of cytokines that they produce. Th1 cells secrete IL-2, IFN-γ and TNF-β while Th2 cells produce the cytokines IL-4, IL-5, IL-6, IL-10 and IL-13. Th2 cells can therefore mediate B lymphocyte activation and antibody production and negatively regulate Th1 responses. In general, Th1 cells are required for protection from intracellular microorganisms while Th2 cells mediate the control of extracellular pathogens and are involved in allergic type reactions.

The search for phenotypic markers that distinguish between Th1 and Th2 cells led to the identification of T1, a cell surface molecule which is expressed on Th2 but not Th1 cells (14, 31). T1, also designated ST2L, DER4, and Fit-1 has previously been isolated as a growth factor and oncoprotein responsive gene (1-4. 6). The T1 gene encodes an orphan receptor belonging to the interleukin-1 receptor family (2, 10). Interestingly, the T1 protein has been shown to exist as both a membrane bound molecule with a cytoplasmic domain and as a soluble secreted protein lacking the transmembrane and cytoplasmic domains (10).

Relatively little is known about T1 signaling and its role in vivo. Whilst T1 is homologous to IL-1R, it does not bind IL-1α and only binds IL-1β very weakly (23, 25, 26). A putative T1 ligand that has been cloned by Gayle et al. has not yet been shown to trigger receptor activation. Kumar et al. isolated two proteins of 18 kDa and 32 kDa that could bind the extracellular domain of T1 and induce NFκB activation. The cloning of these candidate T1 ligands is awaited and the precise functions of T1 remain uncertain. In view of the selective expression of T1 on Th2 lymphocytes, the role of this molecule in Th2 responses has been studied using anti-T1 antibodies or a T1-Ig fusion protein. Administration of an anti-T1 antibody to Leishmania major-infected BALB/c mice was shown to lead to an increased resistance, as revealed by a reduced parasite load and the occurrence of smaller lesions, indicating that the Th2 response was decreased (31). In addition, the severity of collagen-induced arthritis was enhanced when susceptible DBA/1 mice were immunized with type II collagen in Freund’s complete adjuvant (31). Similarly, administration of either an anti-T1 mAb or a T1-Ig fusion protein blocked the production of Th2-specific cytokines and reduced the eosinophilic inflammatory response in a murine adoptive transfer model of Th1- and Th2-mediated lung mucosal immune response (71). Furthermore, in vitro polarization of CD4+ T cells in the presence
of a T1-Ig fusion protein blocked differentiation and activation of Th2 but not Th1 effector cells (71). The above findings therefore suggest that the T1 molecule may be involved in the induction of a Th2 response and that blockade of T1 signaling by an antibody or fusion protein may inhibit the differentiation or activation of Th2 effector cells. However, anti-T1 antibody was also shown to enhance complement-mediated lysis of Th2 cells \textit{in vitro} (31). It is therefore possible that decreased Th2 responses observed \textit{in vivo} following administration of anti-T1 mAb could be due to depletion of T1+ Th2 cells.

In order to investigate the role of T1 in Th2-mediated immune responses we generated mouse strains that contained either a deletion of the T1 gene or constitutively expressed high levels of a T1-Fc fusion protein. In the latter mouse strain, we anticipated that systemic expression of T1-Fc would scavenge the putative T1 ligand and thereby prevent it from binding the T1 receptor. As a model of Th2-mediated protective immunity we used infection with the parasitic nematode \textit{Nippostrongylus brasiliensis}, which induces a strong Th2-type response characterized by high levels of Th2 cytokines, production of IgE and potent eosinophilia in blood and lungs leading to expulsion of the worms (81, 88, 119). Here we demonstrate that neither the absence of T1 nor the overexpression of T1-Fc is essential \textit{in vivo} for the development of a protective Th2 response.
Material and Methods

Tl-knock out targeting construct
The LacZ gene was excised from the plasmid pSVβ (Clonetech) with NoI and subcloned into pBSKS+ (Stratagene). The upstream Tl gene fragment spanning the region between nucleotides 12 and 970 (1 indicates the transcription initiation site at the proximal exon 1) was PCR-amplified with the upstream and downstream primers 5′-TGATCTCGAGCGGCCGCCTGCAGAAATGAGACGAAGG-3′ and 5′ACGCTGGATCCGATTGATGGCTGAGGTCTCTC-3′, respectively. The PCR fragment was cut with BamHI and XhoI and cloned upstream of the LacZ fragment to give rise to pKO2. A 6.8 kb genomic BamHI fragment spanning the region between 5.6 kb and 12.4 kb in the sixth and eighth intron, respectively was subcloned into pBSKS+. The 6.3 kb ClaI fragment was excised and subcloned into the ClaI site of the targeting vector pTK-neo-ums to give rise to pH4A. The 4.5 kb NoI fragment of pKO2 containing the LacZ gene and the upstream Tl sequence was cloned into the NoI site of pH4A. The targeting plasmid pKO4 was linearized with SacII.

Construction of the AAT-Tl-Fc transgene
The Tl-FcIgGγ1 (Tl-Fc) fragment was excised from the previously described plasmid pTl-Hγ1 (30) with the restriction enzymes SacI and BamHI and subcloned into the pBSKS (+) (Stratagene). Subsequently the Tl-Fc fragment was excised with XbaI and XhoI and ligated into the plasmid AAT-8, containing the human α-1 antitrypsin promoter (120). For microinjection the AAT-Tl-Fc sequence was excised from AAT-8 using PvuII, separated by gel electrophoresis, purified using Gel Extraction kit (QIAGEN) and resuspended in 10 mM Tris-HCl pH 8.5 at 100 ng/µl. The fragment was further diluted to a concentration of 2 ng/µl and injected into the pronucleus of fertilized oocytes, derived from the hybrid strain B6C3F1, according to standard methods (121). Transgenic animals were backcrossed with C57BL/6 mice. The animals used for experiments derived from the second, third and fourth backcross with C57BL/6 mice.

Production of Tl-deficient mice
Embryonic stem cells (ES cells) from 129Sv/Ev mice were cultured on mouse embryonic fibroblast feeder layers in DMEM supplemented with 20 % fetal calf serum, 2 mM L-glutamine and 0.1 mM 2-ME. 3 x 10⁷ ES cells were electroporated with 10 µg of
linearized targeting vector pKO4. After 24 h, the culture medium was replaced with selection medium containing 0.2 mg/ml G418 (GibcoBRL, Life Technologies). After 10 days of culture, 500 G418-resistant clones were isolated and monitored for homologous recombination by PCR and genomic Southern blot. One of two clones that contained a disruption of one T1 allele was injected into C57BL/6 blastocysts that were subsequently transferred into pseudopregnant B6CBF1 recipient females. Injection was performed according to standard procedures (RCC, Füllinsdorf, Switzerland). Highly chimeric males were bred to C57BL/6 females. Germline transmission of the mutated T1 gene was suggested by the emergence of offspring with agouti coat color and confirmed by PCR and genomic Southern blot. Mice used in the experiments are homozygous T1-null, heterozygous, and wt offspring of heterozygous parents originating from the first backcross to C57BL/6 mice or homozygous T1-null and wt offspring of the second generation.

**PCR and southern blot analysis**

DNA was extracted from mouse tails by digestion with proteinase K (500 μg/ml) in 50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, and 1% SDS, followed by DNA precipitation with isopropanol. The DNA was resuspended in TE, pH 8.0 and an aliquot used for PCR analysis. Primers used for detection of homologous recombination, as well as LacZ gene insertion were 5’-AAAACTAAAAATTCTATGATGGGCG-3’ (T1-upstream) and 5’-GGATGTGCTGCAAGGCG-3’ (lacZ-downstream), and the downstream primer 5’-GGAAGTGTCAATTGCCA-3’ was used to detect the endogenous T1 gene. For Southern blot analyses 5 μg of genomic DNA were digested with SacI and the blots hybridized with a DIG-labeled 1.9 kb XbaI-HpaI fragment spanning the region between -2.7 and -0.8 kb relative to the transcription initiation site at the proximal exon 1. Primers for the detection of transgene insertion were 5’-GCTTAAATCGGACGAGG-3’ (AAT-Promoter-upstream) and 5’-CTGATCCACGTACTGTCG-3’ (T1-downstream). PCR and Southern blot analyses were performed according to standard methods (122).

**Expression of the soluble T1-Fc protein in transgenic mice**

Liver tissue was homogenized in PBS, supplemented with a cocktail of protease inhibitors (CompleteTM, Boehringer Mannheim, Germany), using a sonicator and cell debris were removed by centrifugation. 40 μg of total protein was separated on an 8%
SDS-PAGE and transferred onto a PVDF membrane (Millipore, Bedford, MA). The membrane was incubated with anti-T1 rabbit polyclonal Ab (7) followed by goat anti-rabbit IgG HRP-labeled antibody (1/7000) (Southern Biotechnology Associates, Inc., Birmingham, AL). The concentration of blood T1-Fc molecules was estimated by ELISA. Plates were coated with anti-T1 mAb (clone DJ8) (10 μg/ml) (12), mice were bled and the serum was added in serial dilutions starting with 1/100 dilution. For detection, a goat anti-human IgG HRP-labeled antibody (1/5000; Southern Biotechnology Associates, Inc.) was used. The standard curve was established using serial dilutions starting with 1/100 dilution. For detection, a goat anti-human IgG HRP-labeled antibody (1/5000) was used. The standard curve was established using serial dilutions (10 μg/ml to 56 ng/ml) of the T1-IgG recombinant molecule (12).

*N. brasiliensis* inoculation and quantitation of worms and eggs

*N. brasiliensis* was maintained by passage through 6-12 week old Lewis rats. Eight to twelve-week old mice were inoculated with third-stage infective larvae by s.c. (500-750 L3) injection. Parasite egg numbers were determined from feces collected daily from individual or groups of mice, as indicated. Adult worm numbers were determined per mouse by removing small intestine, slicing open longitudinally and analyzing microscopically for the presence of worms.

Determination of peripheral blood and tissue eosinophilia

Blood smears were collected on the days indicated, stained with Diff-Quik® (DADE BEHRING AG, Düdingen, Switzerland) and evaluated microscopically as percentage of a total of 200 cells. For determination of lung eosinophil infiltration, a bronchoalveolar lavage (BAL) was performed, cytospins were prepared, stained with Diff-Quik® and a minimum of 200 cells were counted with the percent eosinophils and total eosinophil number/ml of BAL cells calculated.

Isolation of lung lymphocytes and intracellular FACS analysis

Lungs from infected mice were removed on the days indicated, cut into small pieces and incubated for 90 min at 37°C in BSS supplemented with 1 mg/ml DNase and 2 mg/ml Collagenase D. Single cell suspensions were then obtained by shearing the tissue through an 18 G needle. Lymphocytes were separated through a Ficoll gradient and restimulated for 4 h on anti-CD3 mAb-coated plates (10 μg/ml, 2C11) in 10% complete IMDM
supplemented with IL-2 and monensin (2μM). Surface expression of T1 and intracellular production of IL-4, IL-5, and IFN-γ was determined by standard immunofluorescence staining and flow cytometry using the following antibodies: anti-CD4-Tri-Color (Caltag Laboratories, Burlingame, CA), anti-T1-FITC (DJ8) and anti-IL-4-PE, anti-IL-5-PE or anti-IFN-γ-PE (PharMingen). Immunofluorescent-labeled cells were analyzed using a FACScan flow cytometer and CellQuest software (Becton Dickinson, Mountain View, CA).

**Determination of IL-5-secreting cells by ELISPOT**

Lymphocytes isolated from the lungs of infected mice were prepared as described above and restimulated for 10 h with immobilized anti-CD3 mAb (10 μg/ml, 2C11) and IL-2 in the presence of immobilized rat anti-mouse IL-5 mAb (10 μg/ml, TRFK-5, PharMingen). Cells were removed by washing and captured cytokine was detected with rat anti-mouse IL-5 (5 μg/ml, TRFK-4-biotin, (123)) and alkaline phosphatase-conjugated streptavidin (Promega). Spots were developed with 5′BCIP in Alkaline buffer solution (Sigma).

**Determination of cytokines and serum antibody by ELISA**

Lymphocytes isolated from the lungs of infected mice were prepared as described above and restimulated with immobilized anti-CD3 mAb (10 μg/ml, 2C11) and IL-2. Supernatants were removed after 60 h culture and stored at -20°C until assayed. For antibody determination mice were bled from the retro-orbital plexus on the days indicated and total serum IgE and IgG1 levels were determined by ELISA. Briefly, polyvinyl chloride 96-well plates were coated with 5 μg/ml rat anti-mouse IgE (R35-72, PharMingen), 2.5 μg/ml rabbit anti-mouse IgG (Zymed, S. San Francisco, CA), 2.5 μg/ml rat anti-mouse IL-5 (TRFK-5), 2.0 μg/ml rat anti-mouse IL-4 (11B11, (124)) or 5.0 μg/ml rat anti-mouse IFN-γ (AN-18.17.24, (125)) mAb. Plates were blocked with 4% BSA in PBS for 2 h. Dilutions of serum or culture supernatants and standards were added, incubated for 2 h at room temperature and bound immunoglobulin or cytokine was detected with 2.5 μg/ml rat anti-mouse IgE (R35-118-biotin, PharMingen), 1 μg/ml rabbit anti-mouse IgG1-HRP (Zymed), 0.5 μg/ml rat anti-mouse IL-4 (BVD6-24G2-biotin, PharMingen), 5.0 μg/ml rat anti-mouse IL-5 (TRFK-4-biotin, (123)), or 2.0 μg/ml rat anti-mouse IFN-γ (XMG1.2-biotin, PharMingen) followed by incubation with peroxidase-conjugated streptavidin (Jackson Immuno Research Laboratories, Inc. West Grove, PA) for biotin-labeled antibodies. The reaction was developed with ABTS
(Boehringer Mannheim, Germany) and the OD was read at 405 nm in a Microplate Reader (BioRad Laboratories, CA). Cytokine or total serum antibody concentrations were determined using Microplate Manager III software (BioRad Laboratories, CA) and were calculated using IgE mAb (anti-TNP, clone TIB-141, ATCC), IgG1 mAb (Zymed), or recombinant cytokines (PharMingen) as standards.
Results

Generation of T1-deficient mice

Embryonic stem (ES) cells with one disrupted T1 allele were generated by homologous recombination with the targeting construct pKO4 (Fig. 18A). After selection with G418, 500 clones were screened for homologous recombination by PCR using the primers indicated in Fig. 18A. Two cell clones with a disrupted T1 gene were identified and injected into C57BL/6 blastocysts, yielding 6 chimeric mice. Two highly chimeric males were backcrossed to C57BL/6 females and both males transmitted the mutation through their germ line. 12 of 17 offspring were heterozygous for the T1 gene deletion as revealed by PCR and Southern blot analyses of tail DNA (Fig. 18B). Mating of heterozygous animals yielded wild-type, heterozygous and T1-null offspring in a normal Mendelian ratio. All mice were viable and no obvious phenotypic abnormalities could be detected. To demonstrate the absence of T1 expression in T1-deficient mice, lymphocytes were isolated from lungs of N. brasiliensis-infected mice and analyzed for surface expression of T1 by flow cytometry. A CD4+/T1+ cell population was observed in lymphocytes of wild-type mice but not of T1-deficient mice (Fig. 18C).
Figure 18
Figure 18. Disruption of the T1 Gene.
A) Targeting strategy. The genomic organization of a part of the T1 locus is shown in the first line with exons 1 - 10 depicted by the filled boxes. The linearized targeting construct pKO4 is depicted in the second line with the sites used for cloning indicated. The construct harbors a 1 kb and a 6.7 kb genomic T1 fragment flanking the LacZ and the neomycin resistance (neo) genes. The herpes simplex thymidine kinase (HSV-tk) gene is located downstream of the longer T1 fragment. The LacZ gene was placed under control of the T1 promoter. The third line represents the targeted T1 locus where 4.8 kb of the endogenous T1 locus, including part of exon 2 and all of the exons 3 to 6, were deleted and replaced by the LacZ gene. Arrows indicate primers used for PCR to test homologous recombination. B) Southern blot analyses. Genomic DNA was digested with SacI and hybridized with a 1.9 kb DNA fragment derived from the region upstream of exon I as indicated in Fig. 18A. The 9.7 kb and 7 kb bands are indicative for the wild-type and mutant allele, respectively. Molecular size markers are shown in the first lane (M). C) FACS analysis demonstrating the absence of T1 protein in T1-deficient mice. Flow cytometric analysis was performed on lymphocytes isolated from lung tissue of N. brasiliensis-infected T1-deficient (-/-) and wild-type control littermates (+/+), double-stained with Tri-Color-conjugated anti-CD4 mAb and FITC-labeled anti-T1 mAb.
Generation of T1-Fc transgenic mice

In an attempt to scavenge the putative T1 ligand and thereby prevent its interaction with the T1 receptor on lymphocytes, we generated transgenic mice that produced a soluble form of T1 in the liver and secreted it into the blood stream. A cDNA encoding the extracellular domain of T1 fused to the Fc portion of human IgGyl was placed under control of the liver-specific promoter of the α-1 antitrypsin (AAT) gene (Fig. 19A). The transgene was injected into the pronucleus of fertilized eggs and six founder mice were obtained. The insertion of the transgene was confirmed by PCR and Southern blot analyses (data not shown). Expression of the fusion protein in the liver was tested by Western blot analyses using affinity-purified T1 antiserum (Fig. 19B), and the presence of soluble T1-Fc in the blood was detected by ELISA using an anti-T1 mAb (Table 1). One transgenic line with a strong transgene expression was selected for further experiments (Fig. 19B, Table 1). The protein levels in this line ranged from 0.13 - 0.21 mg/ml in hemizygous and around 0.4 μg/ml in homozygous mice. No T1-Fc expression was detected in the blood of non-transgenic littermates (Table 1).
Figure 19. T1-Fc Transgene expression.
A) Transgenic construct AAT-T1-IgG used to generate T1-Fc transgenic mice. sT1 designates the extracellular region of T1 and arrows indicate the position of primers used for PCR to detect transmission of the transgene in the progeny of transgenic founder mice. B) Immunoblot of the T1-Fc fusion protein expressed in the liver of transgenic animals. Whole liver homogenates of T1-Fc transgenic mice were subjected to Western blot analysis using affinity purified rabbit anti-T1 antisera as primary and HRP-conjugated anti-rabbit Ab as secondary antibodies. One of six transgenic lines, designated TgN(AATsT1Fc)423Zbz, accumulated detectable levels of the T1-Fc fusion protein in the liver (lane 7). The molecular weight of the protein (97 kDa) corresponds to that of a T1-Fc fusion protein produced in tissue culture cells and shown in the first lane.

Table 1. Detection of T1-Fc fusion protein in the serum of transgenic animals

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>Genotype</th>
<th>Transgene expression (μg/ml serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg 423.1</td>
<td>non-Tg</td>
<td>0</td>
</tr>
<tr>
<td>Tg 423.2</td>
<td>2n</td>
<td>448</td>
</tr>
<tr>
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<td>n</td>
<td>216</td>
</tr>
<tr>
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<td>n</td>
<td>147</td>
</tr>
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<td>n</td>
<td>189</td>
</tr>
<tr>
<td>Tg 423.6</td>
<td>n</td>
<td>133</td>
</tr>
</tbody>
</table>

*ELISAs were performed using anti-T1 mAb and HRP-conjugated anti-human IgG as capture and detecting antibodies, respectively.

*Non-transgenic littermates (non-Tg), hemizygous (n) and homozygous (2n) animals for transgene insertion.
IL-5 production after *N. brasiliensis* infection is slightly altered in T1-deficient and T1-Fc transgenic mice

In order to investigate the capacity of T1-deficient or T1-Fc transgenic mice to mount Th2 responses, specific pathogen free mice were infected with the nematode *N. brasiliensis* and lymphocytes from lung tissue were isolated 9, 12 or 15 days post infection. Isolated lymphocytes were stimulated *ex vivo* with plate-bound anti-CD3 mAb in the presence of monensin for 4 h and cytokine production and T1 expression was determined by FACS analysis. The proportion of IL-5^+^ CD4 T cells in T1-deficient mice was found to be slightly decreased on day 12 post infection as compared to control littermates (Fig. 20A). A similar decrease in IL-5^+^ CD4 T cells was observed in the T1-Fc transgenic mice on day 9 post infection (Fig. 20B). The decrease in IL-5 production was confirmed by ELISPOT analysis of lung lymphocytes stimulated with anti-CD3 mAb for 10 h (Fig. 20C, D) and ELISA analysis of culture supernatants obtained from lung lymphocytes stimulated *in vitro* for 60 h (Fig. 20E, F). In contrast, the proportion of IL-4-producing CD4^+^ T cells was similar in all groups of mice and varied between 2 and 10% of CD4^+^ cells (data not shown). An increase in T1 expression on CD4^+^ cells, reaching a peak on day 12 post infection, was observed in T1-Fc transgenic mice and both groups of control mice (data not shown). Single cell suspensions were also prepared from the draining lymph nodes (mediastinal and mesenteric) of *N. brasiliensis*-infected mice and cells were re-stimulated *in vitro* with immobilized anti-CD3 mAb and IL-2. The supernatant was collected after 60 h incubation and cytokine production was determined by ELISA. In both the mediastinal and mesenteric lymph node cells, the production of IL-4, IL-5, and IFNγ was similar in both T1-deficient and T1-Fc transgenic mice as compared to their control littermates (data not shown). Production of neither IL-5 nor IL-4 could be detected from lymphocytes obtained from non-infected mice (data not shown). Thus, the absence of the T1 molecule has only a minor influence on the development of Th2 responses in this model of anti-parasite immunity.
T1-deficient T1-Fc Tg

% IL-5 CD4+ cells

Day post infection

Figure 20
Figure 20. Cytokine production by isolated lung lymphocytes from *N. brasiliensis* infected mice.

(A, B) FACS analysis. Lymphocytes from the lungs of T1-deficient (A) and T1-Fc transgenic (B) mice were isolated on day 9, 12 and 15 after infection with *N. brasiliensis* and restimulated on anti-CD3 mAb-coated plates in the presence of monensin for 4 h. Lymphocytes were first double stained with anti-CD4-Tri-Color and anti-T1-F1TC for detection of cell surface expression, followed by permeabilization and staining with anti-IL-5-PE for detection of intracellular cytokine expression. The gate was set on CD4+ cells and the percentage of cytokine-producing CD4+ lymphocytes is indicated. (C, D) ELISPOT analysis of IL-5-producing T cells. Lung lymphocytes from T1-deficient (C) and T1-Fc transgenic (D) mice were restimulated *in vitro* with immobilized anti-CD3 mAb and IL-2 for 10 h in the presence of immobilized anti-IL-5 mAb as capture antibody. The number of IL-5-producing cells per total number of lymphocytes is indicated. (E, F) Measurement of IL-5 production by ELISA. Lung lymphocytes from T1-deficient (E) and T1-Fc transgenic (F) mice were restimulated *in vitro* with immobilized anti-CD3 mAb and IL-2 for 60 h and the supernatant analyzed for the presence of IL-5 by ELISA. Open bars indicate T1-deficient (C, E) and T1-Fc transgenic (D, F) mice, respectively and black bars indicate control mice.
Effects of absence of T1 and T1-Fc overexpression on peripheral and lung eosinophilia

Infection of mice with *N. brasiliensis* leads to the induction of peripheral blood and lung eosinophilia, which is dependent on IL-5 production (88). Since there was a consistent difference in IL-5 production by the lung lymphocytes from the T1-deficient and T1-Fc-transgenic mice, we investigated whether eosinophil levels in the peripheral blood or in the lung were also affected. Infection of both T1-Fc transgenic and T1-deficient mice led to an increase in peripheral blood eosinophil levels to 12-15% of total blood cells. Although a slightly higher percentage of eosinophils were consistently observed in the control mice as compared to both the T1-deficient and T1-Fc transgenic mice, the variability of eosinophil levels between individual mice was quite high and thus the observed differences were not significant (Fig. 21A, B).

Because *N. brasiliensis* infection also leads to eosinophil recruitment into lung tissue, cells from the lungs of infected mice were collected by bronchoalveolar lavage (BAL) at different time points post infection. While mice deficient in the T1 molecule show no difference in the amount of eosinophils recruited to the lung tissue with respect to their control group (Fig. 21C), the T1-Fc transgenic mice exhibit a consistent decrease in the number of eosinophils in the BAL fluid compared to their non-transgenic littermates (Fig. 21D). At day 12 post infection, the T1-Fc transgenic mice exhibit more than a twofold decrease in eosinophils compared to control animals (Fig. 21D).
Figure 21. Eosinophil numbers in peripheral blood and lung after *N. brasiliensis* infection.

(A, B) Peripheral blood eosinophilia. T1-deficient (A) and T1-Fc transgenic (B) mice and control littermates were inoculated s.c. with 750 L1 *N. brasiliensis* larvae. Blood smears were prepared from peripheral blood on the days indicated, stained with Diff-Quik, and the percentage of eosinophils determined by counting a minimum of 200 cells. The given values represent the mean percent of eosinophils from three mice ± SD and results are representative of three separate experiments. (C, D) T1-deficient (C) and T1-Fc transgenic (D) mice and control littermates were inoculated as above and BAL cells were collected on the days indicated, centrifuged onto glass slides, stained and counted as above. A solid black (control animals) or gray (transgenic animals) bar indicates the mean eosinophil number at each time point. Results shown are representative of three experiments, analyzing two to three mice per group.
Total serum IgE and IgG1 levels in *N. brasiliensis* infected mice are not altered in T1-deficient or T1-Fc transgenic mice

Nematode infection typically induces antibodies of the IgE and IgG1 isotypes. IgE expression is dependent on IL-4 whereas IgG1 production is not (76, 79, 126). We assayed total IgE and IgG1 levels in the serum of control, T1-deficient and T1-Fc transgenic mice following infection with *N. brasiliensis*. IgE levels increased in the all mice tested in a comparable fashion, peaking on day 15 post infection and decreasing slowly thereafter (Fig. 22A, B). Total IgG1 levels also increased markedly in T1-deficient, T1-Fc transgenic and control littermates following infection with *N. brasiliensis* and no differences were observed between the different mice (Fig. 22C, D).

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**Figure 22. Serum IgE and IgG1 levels after *N. brasiliensis* infection.**

T1-deficient (A, C) samples were collected on the indicated days post infection. The serum was analyzed for total IgE (A, B) and IgG1 (C, D) by ELISA as described in materials and methods. A solid black (control animals) or gray (transgenic animals) bar indicates the mean level of antibody production at each time point. Blood was collected from 2-5 animals per group and results shown are representative of three separate experiments.
T1-deficient and T1-Fc transgenic mice clear *N. brasiliensis* infection with normal kinetics

In a normal *N. brasiliensis* infection of immuno-competent mice, parasite eggs can be detected in the feces 5 to 6 days after infection, with the peak of egg production observed approximately 9 days post infection (127). This is then followed by a sharp decline in egg numbers just before the adult worms are expelled from the host, around day 12 post infection (77). It is thought that this reduction in egg numbers is due to host-mediated parasite damage resulting in less egg production by the adult worms in the intestine. Therefore, in order to examine whether T1 has an influence on the anti-*N. brasiliensis* immune response the number of parasite eggs present in the feces of infected T1-deficient and T1-Fc transgenic mice was determined. Feces were collected daily from the infected mice starting at day 5 or 6 post infection and parasite egg counts determined. As depicted in Figure 23, both the T1-deficient (Fig. 23A) and the T1-Fc transgenic mice (Fig. 23B) displayed no significant difference in parasite egg production compared to control littermates.

In addition to parasite egg production, the size and characteristics of the adult worm population found in the small intestine at peak infection is often used as an indicator of the efficacy of the protective immune response. Therefore, as an additional readout of the requirement for T1 expression in a protective Th2 immune response, the number of adult worms resident in the small intestine on days 6, 9, and 12 post infection was analyzed. We found that although the amount of adult worms recovered was somewhat variable between individual mice, there was no significant difference in worm burden between T1-deficient or T1-Fc transgenic mice and their control littermates (data not shown). In addition, all mice successfully cleared the adult worms with the same kinetics (data not shown). These results indicate that protective immunity against *N. brasiliensis* infection can occur independently of the expression of T1.
Figure 23. Parasite egg numbers after N. brasiliensis infection. T1-deficient (A) and T1-Fc transgenic (B) mice were inoculated s.c. with 750 L1 larvae and the number of parasite eggs per gram feces determined from daily fecal collections of five individual mice per group. Results represent the mean ± SEM and are representative of three separate experiments.
Discussion

In the present study, we generated T1-deficient and T1-Fc transgenic mice and infected them with the nematode parasite *N. brasiliensis* in order to investigate the role of the T1 molecule in the development of a Th2 response. We found that a normal protective Th2 response could develop in the complete absence of the T1 protein (T1-deficient mice). Similarly, a protective Th2 response developed normally in the T1-Fc transgenic mice where a soluble form of the T1 molecule fused to the Fc portion of human IgG1 is present in large amounts in the serum. Presumably this T1-Fc fusion protein would bind to the putative ligand of the T1 molecule and potentially block its interaction with T1 on Th2 cells. We therefore conclude that ligation of T1 on Th2 cells is not required for the development and effector function of Th2 cells *in vivo*, at least not in response to infection with *N. brasiliensis*.

Expression of T1 has been reported to be exclusively on Th2 and not Th1 cells (14, 31, 32). During the course of a *N. brasiliensis* infection we analyzed the level of T1 expression on CD4+ cells isolated from the lungs of infected mice. We found that T1 expression was very low or undetectable at early time points (day 3) post infection or on cells isolated from lymph nodes that do not drain any site of infection. However, T1 expression increased greatly and was clearly detectable on 8 - 10% of the CD4+ T cells isolated from the lung tissue at the peak of infection (data not shown). A large fraction of T1+ CD4+ T cells were also positive for IL-4 or IL-5. These cells did not produce IFN-γ as revealed by intracellular FACS analysis, indicating that they were Th2-type cells. We therefore confirm earlier reports demonstrating the exclusive expression of T1 on Th2 cells in *in vitro* T cell polarization experiments and *ex vivo* from Th2-dominated responses (31, 32). Nevertheless, IL-4- and IL-5-secreting cells still developed in the absence of T1 or in the presence of T1-Fc. Although we report that protective immunity against *N. brasiliensis* was intact in both the T1-deficient and T1-Fc transgenic mice, we found that the proportion of IL-5-producing Th2 cells isolated from infected lungs was slightly decreased. This decrease in IL-5 production was reflected in the decrease in eosinophil infiltration in the BAL fluid, especially evident in the T1-Fc transgenic animals. Interestingly, it has previously been reported that administration of an anti-T1 antibody or a recombinant T1 fusion protein attenuated a Th2-mediated eosinophilic lung inflammation in mice (32, 71). In addition, spleen cells obtained from IL-5-deficient mice have been reported to have a 3-fold lower expression of T1 compared to IL-5+/−
controls (32). Therefore, it is possible that regulation of IL-5 production and T1 expression may be interdependent. Nevertheless, differentiation into IL-5-producing Th2 cells could still occur in the absence or blockade of T1, and T1 expression can still occur in the complete absence of IL-5 (32), indicating that the counter-regulatory effects of these two molecules are not absolute and other molecular interactions may also play a role.

Recently, another group generated a T1-deficient mouse strain and investigated Th2 cell development in vivo (128). In the present study, we confirm their results and extend them to demonstrate that T1-Fc transgenic mice are not affected in the development of a Th2 immune response. Extensive analysis of the Th2 response induced by infection with N. brasiliensis revealed a possible link between optimal IL-5 production and T1 expression on Th2 cells. In conclusion, T1 appears to be expressed on Th2 cells in vivo but ablation of T1 expression or systemic accumulation of T1-Fc protein seems not to have a profound effect on the development of a protective Th2 response and may therefore limit its potential as a target molecule for therapeutic manipulation of Th2 responses.
Additional Results

**IL-4 production after N. brasiliensis infection is not altered in Tl-deficient and Tl-Fc transgenic mice**

Like IL-5 production, IL-4 secretion of CD4+ T cells is indicative of an ongoing Th2 response. Therefore, IL-4 production was also measured following infection with *N. brasiliensis*. Lung lymphocytes of *N. brasiliensis*-infected Tl-deficient or Tl-Fc transgenic mice were isolated, stimulated as described above and analyzed for IL-4 production. FACS analyses revealed that the mean of the number of IL-4-producing CD4+ T cells was slightly decreased in Tl-deficient and Tl-Fc transgenic mice (Fig. 24A, B). However, individual mice within each group displayed great variability in the amount of IL-4 producing CD4+ T cells and therefore the differences observed were not significant. ELISPOT analysis of lung lymphocytes stimulated with anti-CD3 for 10 h also showed lower numbers of IL-4-secreting CD4+ T cells, especially in Tl-deficient mice on day 12 post infection. However, once again there was large variability within the groups analyzed and therefore, although the lower mean may indicate a tendency for decreased IL-4 production, the differences were not significant (Fig. 24C, D). In addition to FACS and ELISPOT analysis to estimate the number of IL-4 producing cells, the amount of IL-4 produced and secreted into the culture supernatant after 60 h of *in vitro* restimulaiton was measured by ELISA. As depicted in Fig. 24E, F there was no difference between control mice and Tl-deficient or Tl-Fc transgenic mice in the amount of IL-4 produced. Some fluctuation of IL-4 production among individual mice within each group was observable, but the mean IL-4 levels were comparable. Thus, IL-4 production by lung lymphocytes of *N. brasiliensis*-infected mice is not significantly affected by the absence of the Tl protein or of its putative ligand.
Figure 24
Figure 24. IL-4 cytokine production by isolated lung lymphocytes from *N. brasiliensis*-infected mice.

(A, B) FACS analysis. Lymphocytes from the lungs of T1-deficient (A) and T1-Fc transgenic (B) as well as from wild-type mice were isolated on day 9, 12 and 15 after infection with *N. brasiliensis* and restimulated on anti-CD3 mAb-coated plates in the presence of monensin for 4 h. Lymphocytes were first double stained with anti-CD4-Tri-Color and anti-T1-FITC for detection of cell surface expression, followed by permeabilization and staining with anti-IL-4-PE for detection of intracellular cytokine expression. The gate was set on CD4+ cells and the percentage of cytokine-producing CD4+ lymphocytes is indicated. Each symbol represents one animal and the bars indicate the mean value. (C, D) ELISPOT analysis of IL-4-producing T cells. Lung lymphocytes from T1-deficient (C), T1-Fc transgenic (D), and wild-type mice were restimulated in vitro with immobilized anti-CD3 mAb and IL-2 for 10 h in the presence of immobilized anti-IL-4 mAb as capture antibody. The number of IL-4-producing cells per total number of lymphocytes is indicated. (E, F) Measurement of IL-4 production by ELISA. Lung lymphocytes from T1-deficient (E), T1-Fc transgenic (F), and wild-type mice were restimulated in vitro with immobilized anti-CD3 mAb and IL-2 for 60 h and the supernatant analyzed for the presence of IL-4 by ELISA. Open bars indicate individual T1-deficient (C, E) and T1-Fc transgenic (D, F) mice, respectively and black bars indicate single control mice.
**T1 expression on CD4+ T cells after infection with *N. brasiliensis***

It has previously been shown that the percentage of T1-positive T cells increases under Th2 polarizing conditions following *in vitro* restimulation with antigen (71). To test the level and course of T1 expression on CD4+ T cells *in vivo* in a model system, lymphocytes from lungs of *N. brasiliensis*-infected mice were isolated at different time points after infection and restimulated *in vitro* with anti-CD3 mAb for 4 h in the presence of IL-2 and monensin. FACS analysis revealed that T1 expression was very low or undetectable at early time points (day 6), but increased continuously, reaching a peak in T1-Fc transgenic mice and both groups of control mice on day 12 post infection (Fig. 25). Whether this increase results from an enhanced T1 expression on cells already present in the lungs or reflects an increased recruitment of T1 expressing Th2 cells into the lung tissue remains to be determined. By comparing the total number of lymphocytes isolated from the lungs there was a small but consistent increase in cell number with a peak around day 9 and around day 12 after infection in T1-Fc transgenic and T1-deficient mice, respectively. In both T1-Fc transgenic and control mice there is a decline in cell number thereafter. This observation would favor the model that activated T1 expressing lymphocytes are recruited into the lung tissue. However, no significant differences were observed in the total cell number of isolated lung lymphocytes in T1-deficient and wild-type mice, indicating that similar amounts of CD4+ T cells are recruited independently of T1 expression. In addition, intracellular cytokine staining revealed that a large proportion of these CD4+ T cells stained positive for IL-4 or IL-5, but not IFN-γ, revealing that they are Th2-type cells. Similarly, a large proportion of T1'CD4+ T cells were also positive for IL-4 and IL-5, but not IFN-γ, indicating that the T1 expressing cells are almost exclusively Th2-type cells (data not shown).
Figure 25. T1 expression on CD4⁺ T cells isolated from lungs of *N. brasiliensis*-infected mice.
Lymphocytes from the lungs of T1-deficient (A) and T1-Fc transgenic (B) mice were isolated on day 6, 9, 12 and 15 after infection with *N. brasiliensis* and restimulated on anti-CD3 mAb-coated plates in the presence of monensin for 4 h. Cells were double stained with anti-CD4-Tri-Color and anti-T1-FITC for detection of cell surface expression. The gate was set on CD4⁺ cells and the percentage of T1-expressing CD4⁺ lymphocytes is indicated. Each symbol represents the value of an individual animal. This experiment was repeated three times with similar results.

IL-4, IL-5, IL-13, and IFN-γ production in the mediastinal, mesenteric and inguinal lymph nodes
To evaluate whether Th2 cell effector functions were altered in the absence of the T1 protein or T1 ligation, IL-4, IL-5, IL-13 and IFN-γ production in mediastinal, mesenteric and inguinal lymph nodes were analyzed. We chose to investigate the cytokine production from these lymph nodes because the mediastinal lymph nodes drain the lungs and the mesenteric lymph nodes the small intestine, both are sites that are invaded by the parasite during the course of infection, whereas the inguinal lymph nodes do not drain any site of infection and reveal only background levels of cytokine production. Lymph nodes of *N. brasiliensis* infected T1-deficient or T1-Fc transgenic mice were isolated and single-cell suspensions were restimulated *in vitro* with immobilized anti-CD3 mAb and IL-2. Supernatants were collected after 60 h incubation and IL-4, IL-5, IL-13 and IFN-γ levels measured by ELISA (Fig. 26). While IL-4, IL-5, IL-13 are typical Th2 type cytokines, enhanced IFN-γ production is indicative for a shift towards a Th1 response. Lymphocytes obtained from the mediastinal lymph nodes showed a peak in production of all four cytokines around day 6 post infection and no differences were observed between T1-deficient or T1-Fc transgenic and control mice. Lymphocytes from the mesenteric
lymph nodes showed the highest IL-4, IL-5 and IL-13 secretion around day 9 which is in accordance with the course of infection as the *N. brasiliensis* larvae initially travel through the lung and are subsequently coughed up and swallowed thus gaining access to the small intestine. These cytokines, especially IL-4 and IL-13 are crucial for the induction of type 2 inflammation, host protection and expulsion of the worm (75, 85, 129). While the production of these Th2 type cytokine increases, the IFN-γ production decreases to background level. Low amounts of cytokine production were observed in the non-draining inguinal lymph nodes or in lymph node cells of non-infected mice (data not shown). Since the production of all cytokines tested was similar in both T1-deficient and T1-Fc-transgenic mice as compared to their wild-type controls, it seems that the absence of the T1 molecule or the lack of T1 signaling does not affect the cytokine production in the draining lymph nodes.
IL4 producing mediastinal LN cells

IL4 producing mesenteric LN cells

Figure 26 A
IL-5 producing mediastinal LN cells

IL-5 producing mesenteric LN cells

IL-5 producing inguinal LN cells

Day post infection

Figure 26 B
IL-13 producing mediastinal LN cells

IL-13 producing mesenteric LN cells

IL-13 producing inguinal LN cells

Day post infection

Figure 26 C
IFN-γ producing mediastinal LN cells

IFN-γ producing mesenteric LN cells

IFN-γ producing inguinal LN cells

Figure 26 D
Figure 26. Cytokine production of mediastinal, mesenteric and inguinal lymph nodes.
(A-D) After infection with *N. brasiliensis*, lymph nodes of T1-deficient, T1-Fc transgenic and wild-type mice were taken on the days indicated. Single-cell suspensions were prepared and the cells were restimulated *in vitro* with immobilized anti-CD3 mAb and IL-2. Supernatants were collected after 60 h incubation and IL-4 (A), IL-5 (B), IL-13 (C) and IFN-γ (D) levels determined by ELISA as described in Materials and Methods. Each bar represents the mean ± SEM concentration of cytokine produced from 1x10⁶ lymph node cells from three animals and results shown are representatives of two to four separate experiments.
4.4 Viral infection models

Effective control of acute viral infection is usually mediated by the combination of humoral and cellular immune responses. Thus, T lymphocytes play an important role in immunity against viruses. Here we studied whether the lack or the overexpression of T1 has effects on several parameters of antiviral immunity against lymphocytic choriomeningitis virus (LCMV) and vesicular stomatitis virus (VSV) infections. For noncytopathic viruses such as LCMV, CD8+ CTLs are the major effectors in the eradication of primary infection, while neutralizing antibodies may assist in protecting against reinfection (130). In contrast, for cytopathic viruses such as VSV, T-cell dependent cytokines together with neutralizing antibodies are usually essential for virus elimination and protection against reinfection (103).

4.4.1 Infection with lymphocytic choriomeningitis virus (LCMV)

Injection of replicating LCMV into the hind footpad of a mouse induces a CD8+ T cell-mediated swelling reaction which peaks on day 8 followed by a CD4+ T cell-mediated swelling which peaks on day 11. This primary footpad swelling reaction is mediated by class I-restricted cytotoxic CD8+ followed by class II-restricted helper CD4+ T lymphocytes (95).

To examine whether the T1-deficient or the T1-Fc-transgenic mice show differences in the swelling reaction or in its kinetic compared to wild-type controls, mice were infected with 200-300 pfu LCMV-WE in the left and right hind footpad. The dorsoventral thickness of both hind feet was measured before viral challenge and from day 6 p.i. until the swelling reaction started to decline. As shown in Fig. 27A, B all mice exhibited an increase in footpad thickness, peaking around day 7-8 after injection and declining thereafter. As for T1-Fc transgenic mice, it seems that the decline after the peak of the CD8+ T cell mediated swelling reaction is slightly accelerated as compared to their controls. Therefore, to further investigate the CD4+ T cell-mediated part of the footpad swelling reaction the experiment should be extended by titrating down the viral dose.
LCMV elimination starts around day 7 p.i. and is mediated by virus-specific cytotoxic CD8⁺ T cells. However, this initial virus control is incomplete and LCMV persists at low levels at peripheral sites, from which it may sporadically spread through the circulation. For long-term virus control CD4⁺ T cells and B cells are indispensable (101). While neutralizing antibodies appear relatively late (>60 days) after virus infection and are directed against the viral glycoproteins (131), non-neutralizing antibodies directed against the LCMV nucleoprotein (NP) appear early after infection but seem to be dispensable for viral clearance. Despite their neglectable role in virus elimination, sera of LCMV-infected mice were tested for the presence of NP-specific antibodies by ELISA. T1-deficient and T1-Fc-transgenic mice as well as their littermate control mice accumulate NP-specific antibody titers to comparable levels and with similar kinetics (Fig. 28). This observation rules out the probability that the T1 protein or its ligation influences antibody production early after infection.
Figure 28. Titers of LCMV NP-specific antibodies.

Blood was taken on the days indicated and the serum subjected to ELISA analysis on plates coated with LCMV-NP. Bound IgG was detected with HRP-conjugated goat anti-mouse IgG. Each symbol represents the value of one mouse. (A) Antibody titer of T<sup>1</sup>-deficient (open circles) and wild-type (closed circles) mice, (B) antibody titer of T<sup>1</sup>-Fc transgenic (open triangles) and wild-type (closed triangles) mice. The titer indicates the dilution of the serum that resulted in an OD value which is two times above background.

To address the question whether the absence of the T<sub>1</sub> protein or its overproduction has an effect on the production of neutralizing antibodies, serum was taken on day 58 and 70 p.i. and tested for its neutralizing capacity in a neutralization assay (132). No neutralizing antibodies were found in all mice tested (data not shown). This result is not surprising since previous studies with low-dose LCMV infection into footpads have never resulted in an induction of neutralizing antibody production (L. Hunziker, personal communication). As already mentioned above, neutralizing antibody-producing B cells are of key importance in virus control in the late phase of an acute LCMV infection, but it seems that in a low-dose infection the neutralizing antibody response lies below detectable limits.

4.4.2 Infection with vesicular stomatitis virus (VSV)

VSV infection of immunocompetent mice induces a rapid neutralizing IgM response that around day 6-8 p.i. is switched to a T cell-dependent neutralizing IgG response (133). While the former occurs independently of T cell help, the production of neutralizing IgG antibodies is dependent on CD<sup>4+</sup> T cell help (102). To examine the ability of T<sup>1</sup>-deficient and T<sup>1</sup>-Fc transgenic mice to control VSV infection, we immunized mice intravenously with 2 x 10<sup>6</sup> pfu of live VSV-Indiana (VSV-IND). As shown in Fig. 29 all mice mounted normal T cell-independent neutralizing IgM as well as CD<sup>4+</sup> T cell-dependent
neutralizing IgG responses. The levels of the antibody titers and their kinetics were similar in the T1-deficient, the T1-Fc transgenic and in wild-type mice. Therefore, it seems that neither the lack of the T1 protein on CD4⁺ T cells nor the presumed absence of T1 signaling in CD4⁺ T cells has an influence on the development of a neutralizing IgG antibody response, at least in the case of a high-dose VSV infection (2x10⁶ pfu).

Figure 29. Neutralizing antibody response of T1-deficient, T1-Fc transgenic and control mice after VSV infection.

On the indicated days blood was taken and serum was analyzed in a neutralization assay for the presence of VSV-IND-neutralizing IgM and IgG. Each point represents the mean titer of three mice ± SEM where the variation is more than one dilution step. Neutralizing antibody titers of T1-deficient and wild-type control mice are depicted in (A) and of T1-Fc transgenic and wild-type control mice in (B).

Although the VSV specific IgG response was comparable in the different tested mice, the quality of the response might be altered. To investigate the CD4⁺ T cell-dependent IgG response, the total amount of the immunoglobulin isotypes IgG1, IgG2a and IgG2b were analyzed by ELISA. While IgG1 and IgG2a antibody titers were only slightly elevated (data not shown), the levels of IgG2b increased markedly after VSV infection. The antibody titers reached similar levels with similar kinetics, irrespective of the genotype of the mouse, thereby ruling out a possible involvement of the T1 protein in the immunoglobuline switching (Fig. 30). (Due to technical problems, it was not possible to perform ELISAs to test for the presence of VSV-specific IgG isotypes. Therefore, these assays show total IgG2b and not VSV-specific IgG2b, although it can be assumed, that the increase in serum IgG2b after VSV infection is mainly attributable to the production of VSV-specific antibodies.)
Figure 30. IgG2b specific ELISA of VSV infected T1-deficient, T1-Fc transgenic and littermate control mice.

Blood was taken on the days indicated and the serum incubated on anti-IgG2b coated plates. IgG2b was detected with an HRP-labeled rabbit anti-mouse IgG2b. Antibody titers of the IgG2b isotype in T1-deficient and littermate control mice (A) and in T1-Fc transgenic and wild-type mice (B). Each symbol represents the mean titer ± SEM of three mice.
4.5 Bacterial infection model with *Listeria monocytogenes*

Murine listeriosis has been used as a model system for studying mammalian host defense against intracellular bacterial pathogens in general. Protection against infection with *Listeria monocytogenes* is mediated by natural and acquired immunologic mechanisms. In normal mice, *L. monocytogenes* infection induces a nonspecific inflammatory response involving neutrophils, NK cells and macrophages during the first 24 to 72 h of infection. This response decreases the bacterial burden to a great extent and is essential for the survival of infected mice (113). To evaluate whether these early innate defenses are affected in T1-deficient mice resulting in altered resistance and/or susceptibility to *L. monocytogenes* infection, mice were inoculated intravenously with a sublethal dose of *L. monocytogenes* (4000 bacteria/mouse). After three, five, six or twelve days, mice were killed and their spleens and livers removed. Numbers of bacteria were determined in organ homogenates after serial dilution and plating on agar plates (Fig. 31).

In three experiments we observed increased resistance in T1-deficient mice at early times of infection. The CFU from spleens were up to ten times lower on day 3 and 5, compared with controls (Fig. 31A-C). However, the difference in bacterial burden between T1-deficient and control mice was less clear in another independent experiments (Fig. 31D). The CFUs reached similar values in T1-deficient and control mice (Fig. 31C). In the liver, the bacterial load was low or undetectable. It has been reported that an i.v. inoculum of *L. monocytogenes* is rapidly removed from the blood by the fixed macrophages of the liver (Kupffer cells). This initial inactivation of *L. monocytogenes* coincides with the accumulation of neutrophils, which play a key role in destroying *L. monocytogenes*-infected hepatocytes at foci of infection. Since most of the liver-associated *L. monocytogenes* are killed over the next 2-6 h (113), it is likely that at this infection dose no or little viable bacteria are found in the liver. In the spleen however, there is no early inactivation of *L. monocytogenes* and a progressive infection in this organ can take place. Neutrophils are not required to restrict the growth of the pathogen during the first day of infection, but are critical for combatting the infection at a later stage to prevent uncontrolled proliferation of *L. monocytogenes* at intracellular and extracellular sites throughout the spleen. On day 6 p.i. there were only few bacteria found in the spleen of both T1-deficient and control mice, and on day 12 p.i. bacteria were completely cleared in almost all mice tested (Fig. 31D). This experiment indicates that there is no strong effect of the absence of T1 on bacterial clearance in the late phase.
Whether there is an effect on the early anti-\textit{L. monocytogenes} defense cannot be answered by this experiment. A possible approach to address this question is to inoculate mice with higher doses of \textit{L. monocytogenes} and monitor the course of listeriosis by determining the bacterial burden in the liver and spleen several times within the first 48 hours after infection.

In addition, analysis of components that are critical for resolving the infection such as macrophages, NK cells or neutrophils might be of interest, but have not been further followed in this study.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure31}
\caption{Bacterial burden in spleen after \textit{L. monocytogenes} infection.}
\end{figure}

T1-deficient mice (open symbols) and wild-type controls (closed symbols) were inoculated \textit{i.v.} with $4 \times 10^3$ living \textit{L. monocytogenes} on day 0. The infection was monitored by counting colony forming-units (CFUs) of \textit{L. monocytogenes} in spleens from two to five mice per group. The figure shows four independently performed experiments. In experiment A) and C) spleens were collected after five days, in experiment B) after three and five days and in experiment D) after six and twelve days. A solid black (control animals) or grey (mutant animals) bar indicates the mean number of CFUs at each time point.
5. General Discussion

This work focussed on the expression and the physiological role of the T1 molecule in vivo. In our studies we generated two different types of genetically altered mice: Firstly, T1-Fc transgenic mice, which contain a transgene coding for a T1-IgG fusion protein. This protein is produced in the liver and secreted into the bloodstream in order to scavenge putative T1-ligand(s), thus preventing interaction with the T1 receptor. Secondly, T1-deficient mice were generated by replacing part of the T1 gene via homologous recombination with a targeting DNA construct, resulting in the absence of the T1 protein.

Since there were no signs of abnormalities regarding development, health and fertility, we furthered our search for a possible function of the T1 molecule at its known expression sites. Primary mast cells isolated from the peritoneum or in vitro cultured bone marrow derived mast cells of T1-deficient mice displayed a typical IgE⁺ and c-kit⁺ phenotype. Additionally, no alterations were observed in morphology, as determined by electron microscopy or in mast cell stainings with alcian blue or safranin. Another cell type that expresses the T1 molecule is the T helper cell type 2. Previous studies have shown that T1 is expressed only on Th2 and not on Th1 cells (31, 32). In addition, several groups have shown that administration of anti-T1 Ab or T1-Fc fusion protein in different model systems has inhibitory effects on Th2 development and effector functions (31, 32, 71). Therefore, it was tempting to investigate the role of T1 in the Th2 response in an established model that evokes a predominately Th2-type response, such as infection of mice with the intestinal parasitic nematode Nippostrongylus brasiliensis.

Two main questions were addressed. First, does the absence of the T1 molecule or the overexpression of its soluble form have an effect on the characteristic of the Th2 response and second, is the protective immunity against this parasitic infection altered? We tested the critical parameters of a Th2 response such as IL-4 and IL-5 production, blood and tissue eosinophilia as well as the serum IgE and IgG1 levels. As discussed in chapter 4.3, the T1-deficient and the T1-Fc overproducing mice showed a comparable immune response to their wild-type controls and all mice cleared the parasite infection with normal kinetics. However, analyzing the cytokine expression pattern of isolated lung lymphocytes from N. brasiliensis-infected mice we observed a slight but consistent decrease in IL-5 production in T1-deficient and T1-Fc-transgenic mice in comparison to their wild-type controls. This difference in IL-5 production did not affect blood
eosinophilia, but recruitment of eosinophils into the lungs was slightly lowered, especially in T1-Fc transgenic mice. Similar findings were recently published by Townsend et al. (134) using a primary pulmonary granuloma model, induced with *Schistosoma mansoni* eggs, that elicits Th2 responses. In this model, a cellular granulomatous response develops around parasite eggs that lodge in the lungs after intravenous injection into mice. While in naive wild-type mice granuloma formation was visible and consisted of eosinophil infiltration around the immobilized eggs, T1-deficient mice failed to develop significant pulmonary granuloma formation and eosinophilic infiltration was not detected after intravenous injection with schistosome eggs. In addition, they found that in a secondary pulmonary granuloma model, that involves presensitization of mice by administration of schistosome eggs intraperitoneally before being challenged with an intravenous inoculation of eggs, draining mediastinal lymph node cells from T1-deficient mice produced significantly reduced levels of IL-4 and IL-5. In the *N. brasiliensis* infection model we also estimated the percentage of IL-4 producing lung lymphocytes and the amount of IL-4 they secreted. No statistically relevant difference was observed. These results show that the absence of T1 on Th2 cells influences the cytokine production of these cells differently at distinct sites. Mutant mice infected with *N. brasiliensis* exhibited a decreased IL-5 secretion by lung lymphocytes but displayed a normal increase in IL-4 and IL-5 production in the draining mediastinal and mesenteric lymph nodes. A possible explanation for this finding is that in this model T1 influences the Th2 cytokine production in a tissue-specific manner resulting in a reduced eosinophil infiltration into the lung tissue of mutant mice. In contrast, presensitized mice injected with *S. mansoni* eggs showed reduced levels of IL-4 and IL-5 in the draining mediastinal lymph node cells. This observation could be ascribed to the nature of the antigen challenge or its route of administration. However, after presensitization the magnitude of generated secondary pulmonary granulomas was similar in T1-deficient and wild-type animals, suggesting that in this model T1 is involved in the early events in the induction of Th2 immune reactions, such as production of IL-4 and IL-5 but may not be essential for the generation of downstream effector functions. Hence, it appears that the affected parameters in the immune response are dependent on the model system used. Furthermore, a shift towards a Th1 response could be excluded, since the IFN-γ production by lung lymphocytes or lymph node cells was not enhanced in the mutant animals using either model system. These data are somewhat in contrast to *in vivo* and *in vitro* experiments showing that T1 signaling is
crucial for differentiation and activation of Th2 effector cells. Previous studies using anti-T1 antibody or T1-Ig fusion protein in various Th1 and Th2 predominated infectious models showed exacerbation and reduction of the disease, respectively (31, 71). Since the attenuation of T1 signaling by antibody or T1-Ig fusion protein affected the Th2 effector mechanisms more than the complete absence of the T1 protein, there might be redundant or compensatory mechanisms in the mutant animals that substitute the function of T1. It is possible that such mechanisms only come into play if the T1 protein is completely absent from the beginning of the development. Alternatively, the reduced Th2-mediated functions observed in the studies with anti-T1 antibody might be a result of complement-mediated elimination of the T1-positive cell population.

During the course of infection, T1 expression on lung lymphocytes increased in both control animals and in the T1-Fc transgenic mice, but in the latter group T1 upregulation was not as extensive as in the control group. This might be due to the absence of the T1 ligand, since the soluble T1-Fc might have captured it before reaching its receptor. It has been shown in other systems that the binding of the ligand to its receptor triggers enhanced receptor expression (i.e. VEGF mediated upregulation of VEGF-receptor). Furthermore, the increase of T1 expressing cells can be ascribed to the upregulation of the T1 molecule on CD4+ T cells already present in the lung or to the enhanced recruitment of T1 expressing Th2 cells into the lung tissue. Since the total amount of lymphocytes recovered from the lungs of infected mice is not lowered in T1-deficient mice, it is likely that other CD4+ T cells can substitute for the T1 positive cell population.

To address the question whether T1 has an effect on the protective immunity against N. brasiiliensis, the egg production and the amount of worms in the intestine were determined. The eggs were cleared on day 12 post infection in the T1-deficient, the T1-Fc overexpressing and the control mice. In several independent experiments there was a slight but consistent delay in the peak of egg production in T1-deficient and T1-Fc overexpressing mice compared to their littermate control. At the same time, the mean number of eggs produced was enhanced in the transgenic animals. This subtle difference could be ascribed to the lower IL-5 production seen in these animals. It had been shown in IL-5 transgenic mice that the intestinal worms were fewer in number throughout infection and were less fecund (135). Furthermore, excess amount of IL-5 leads to a persistent eosinophilia, that can impair migration of parasite and reduce their reproductive success (135, 136). Although eosinophilia seemed only mildly affected in T1-deficient and T1-Fc transgenic animals, it is possible that the higher egg number is a
consequence of a minimally altered immune response, allowing enhancement of the worm fecundity. If there is such an effect, it is only minimal as the worm burden in the intestine is not significantly changed in the different mice tested and varies greatly among different mice examined. In addition, a key element in expulsion of *N. brasiliensis* has been ascribed to mucosal mast-cells and to the mucus producing secretory goblet cells in the parasitized gut epithelium (137), rather than to eosinophils. Eosinophils, however, accumulate in response to IL-5 and seem to be important for the killing of the larvae in the lung (135).

Taken together, the T1 molecule seems not to be mandatory for the development of a protective Th2 response to the parasitic nematode *N. brasiliensis*. This finding is not surprising when taking into account that the T1 protein is only expressed on a subset of CD4+ T cells (32, 72). Therefore, even if the subset of T1 positive Th2 cells were inactive or absent in the mutant animals, an effective Th2 response might still be achieved by the T1 negative subset of Th2 cells. However, considering subtle differences such as IL-5 production and reduced primary pulmonary granuloma in *Schistosoma mansoni*-infected mutant mice, the lack of T1 expression might influence certain parameters of a Th2-type immune response, which are critical for the immunological Th2 response to only some infections and might be manifested in some model systems but not in others.

A functional role of T1 in the immune response against viral infection was studied in mice infected with lymphocytic choriomeningitis virus (LCMV) to evaluate T cell responses and with vesicular stomatitis virus (VSV) to assess antibody responses. Primary infection with the noncytopathic LCMV virus is controlled almost exclusively by CTL (97) whereas in infection with the cytopathogenic VSV virus an early neutralizing Ab response against VSV is needed to prevent lethal encephalitis (138). Though the immunological responses to these infections are dominated by CD8+ T and by B cells we wanted to analyse whether the absence of T1 signaling influences in some unknown way the differentiation of these cell types or whether it affects these immunological responses through novel, unknown mechanisms. However, we found no alterations in the immune parameters tested between T1-deficient, T1-Fc transgenic and control mice. As for LCMV infection this finding was expected, since CD4+ T cells seem to play a minor role in acute virus control but are required for permanent virus control (110). In our experimental setting we were not able to detect neutralizing antibodies, and therefore we did not assess the role of CD4+ T cell help for B cells to produce such
antibodies. In contrast, VSV infection induces a rapid neutralizing IgM response, followed by production of neutralizing IgG Abs that are strictly dependent on CD4+ T cell help (102). But neither the T cell help-independent IgM nor the dependent IgG response are different in T1-deficient and T1-Fc-transgenic mice, leading to the conclusion, that T1 is not necessary for the specific immune response against these viruses.

Extending our search for an involvement of T1 in the unspecific immunity in mice, we challenged the mutant mice with the intracellular bacterium *Listeria monocytogenes* and investigated the capacity of the mice to combat these bacteria. By enumerating viable bacterial load in the spleen and liver we found a tendency to lower titers in the T1-deficient mice. We could exclude that this observation is due to bacterial uptake in the liver and spleen, the primary target site. Neither is the specific immune response, needed to completely clear the bacteria at a late stage of infection affected since complete bacterial clearance was observed in the mutated mice. It remains to be tested whether components of the innate immune response such as macrophages, neutrophils or NK cells are affected. In particular the role of macrophages might be of interest, since it has been recently shown that soluble T1-Fc protein binds to macrophages (F.Y. Liew, personal communication). It must be stressed though, that the difference in bacterial titers was not always clear and therefore might represent an artifact, perhaps due to slightly different immunological states of the mice used in the different experiments. Further analysis and larger experimental groups of mice will be needed to clarify this point.

By comparing these different in vivo infection models it can be assumed that T1 is not essential for the Th2 mediated immune response. However, in T1-deficient mice challenged with *N. brasiliensis* the IL-5 production of lung lymphocytes was slightly reduced and both, IL-4 and IL-5 levels were significantly lowered in response to secondary pulmonary granuloma formation (134). The decrease in IL-5 influenced the recruitment of eosinophils into the lung tissue of *N. brasiliensis*-infected mice. Similarly, the onset of primary lung granuloma formation was severely inhibited in T1-deficient mice. Therefore, it is possible that T1 is important at sites where eosinophils are needed such as the lung to combat the infection. Alternatively, T1 may have unknown functions in Th2 cells or other cell populations, which were not manifested in the tested models. In addition, the identification of the cognate ligand might also reveal a novel function of T1 as well as its role in the Th2 immune response. It is also possible that other immunological responses for which mast cells are important (i.e. allergies) are
suppressed or even enhanced, depending on whether the immunological reaction is beneficial or detrimental to the host. Therefore, it is valuable to test further immunological models and to combine the T1 mutation with other mutations (i.e. IL-1, IL-5) in order to specify the role of T1.
6. Materials and Methods

Cell cultures
NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Mouse embryonic fibroblasts (MEFs) and embryonic stem cells (ES cells) were grown in DMEM containing 20% FCS, 2 mM L-glutamine and 0.1 mM 2-mercaptoethanol (2-ME). HepG2 cells were cultured in RPMI 1640 supplemented with 15% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Lymphocytes were isolated from lungs, spleen or lymph nodes of mice and cultured in RPMI 1640 or Iscove’s modified Dulbecco’s medium (IMDM) containing 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 50 μM 2-ME. Bone marrow derived primary mast cells were grown in IMDM containing GLUTAMX (L-alanyl-L-glutamine) supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME and 2% conditioned culture supernatant from murine IL-3-secreting X63/IL-3 cells (139). Vero cells were maintained in Minimum essential medium (MEM) with 5% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. All cells were grown at 37°C in 5% CO₂. All media are from GibcoBRL, Life Technologies.

T1-knock out targeting construct
The 3.5 kb LacZ gene was excised from the plasmid pSVβ (Clonetech) with NotI and subcloned into pBSKS+ (Stratagene). In order to destroy the 5’ NotI site this plasmid was cut with Xbal which cleaves the DNA in close proximity to the NotI site, treated with T4 DNA polymerase in the presence of dATP followed by S1 nuclease digestion and ligation. The upstream T1 gene fragment spanning the region between nucleotides 12 and 970 (1 indicates the transcription initiation site at the proximal exon 1) was PCR-amplified with the upstream and downstream primers 5’-TGATCTCGAGCGGCCGC- GCCTGCAAGAATGAGACGAAGG-3’ and 5’ACGCTTGATCCGATTGATGGGCTGAGGTCTCTCT-3’, respectively. Sequences in italics highlight the XhoI, NotI and BamHI sites. The PCR fragment was cut with BamHI and XhoI and cloned upstream of the LacZ fragment to give rise to pKO2. A 6.8 kb genomic BamHI fragment spanning the region between 5.6 kb and 12.4 kb in the sixth and eighth intron, respectively was subcloned into pBSKS+. The 6.3 kb ClaI fragment was excised and subcloned into the ClaI site of
the targeting vector pTK-neo-ums to give rise to pH4A. The 4.5 kb NotI fragment of pKO2 containing the LacZ gene and the upstream T1 sequence was cloned into the NotI site of pH4A. The targeting plasmid pKO4 was linearized with SacII, loaded onto an 1% agarose gel and purified using the QIAquick Gel extraction kit (QUIAGEN) according to manufacturer’s protocol. The linearized targeting construct was purified twice with phenol/dichloromethane (1:1), precipitated with ethanol and dissolved in sterile TE buffer at 1 µg/µl. Homologous recombination in the transfected ES cells was detected by PCR. To establish the PCR conditions, a test-targeting construct (pKO3) was made. This construct contains the T1-LacZ gene fragment, but with a prolonged T1 gene sequence upstream of exon 1 (290 bps). The upstream primer for amplifying this sequence is 5’-TGATCTCGACGGCGCCATTTGGGAATGGACATTATGG-3’ whereas the downstream primer is identical to the primer described above. The XhoI and NotI site are marked in italics. This PCR fragment was subcloned upstream of the LacZ gene, and the resulting T1-LacZ fragment was inserted into the NotI site of pTK-neo-ums.

Production of T1-deficient mice
Embryonic stem cells (ES cells) from 129Sv/Ev mice were cultured on irradiated (3000 rad) mouse embryonic fibroblast feeder layers in DMEM supplemented with 20% fetal calf serum, 2 mM L-glutamine and 0.1 mM 2-ME. 3 x 10^7 ES cells were trypsinised, washed, resuspended in PBS and electroporated with 10 µg of the linearized targeting vector pKO4 at 500 µF, 240 V (6ms) in a BioRad Gene Pulser. The cells were then transferred to culture medium and plated onto 10 cm² tissue culture dishes containing irradiated feeder cells. After 24 h the culture medium was replaced with selection medium containing 0.2 mg/ml G418 (GibcoBRL, Life Technologies). After 10 days of culture, 500 G418-resistant clones were isolated and monitored for homologous recombination by PCR and genomic Southern blot. Plates containing the ES cells were washed with PBS and then serum-free medium was added. Individual cell colonies, consisting of approximately 10’000 cells, were picked and transferred into a 96-well plate containing 25 µl PBS/well. After picking the desired amount of colonies, 75 µl trypsin/well were added and the cells incubated at 37°C for 3.5 min. Subsequently, 90 µl G418-containing medium was added and the cells resuspended by pipetting up and down ten times. 100 µl of the cell suspension were transferred to a 48-well plate containing feeder cells and the remaining 100 µl were used for PCR screening. Two clones that contained a disruption of one T1 allele were expanded gradually from a 48-well plate to a
T75 cm² flask, whereby the G418 was omitted during the last three passages. The cells were injected into C57BL/6 blastocysts that were subsequently transferred into pseudopregnant B6CBF1 recipient females. Injection was performed according to standard procedures (RCC, Füllinsdorf, Switzerland). Highly chimeric males were bred to C57BL/6 females. Germline transmission of the mutated Tl gene was suggested by the emergence of offspring with mosaic coat color and confirmed by PCR and genomic Southern blot analyses. Mice used in the experiments were homozygous Tl-null, heterozygous, and wild-type offspring of heterozygous parents originating from the first backcross to C57BL/6 mice or homozygous Tl-null and wild-type offspring of the second and third generation.

**PCR to detect homologous recombination with targeting construct pKO4 in ES cells**

This PCR protocol was established with ES cells that were transfected with the test-targeting construct pKO3. Since this construct contains that portion of the 5' flanking region of the Tl gene that was to be used for the detection of homologous recombination of the targeting construct pKO4 with the chromosomal DNA, PCR conditions could be established with cell clones that had randomly integrated pKO3 DNA. pKO3-transfected ES cell clones were isolated, suspended, and 100 µl of this suspension were transferred into a PCR tube and centrifuged at 14’000 rpm for 5 min. All but 5 µl of the supernatant was discarded and the pellet resuspended in 20 µl of 1.25x PCR buffer. This suspension was overlaid with mineral oil and incubated at 95°C for 10 min to inactivate DNases. Proteins were digested with 1 µl proteinase K (10 mg/ml) at 56°C for 1 h, and proteinase K was subsequently inactivated for 10 min at 95°C. 25 µl PCR reaction mix was added to the same tube, adding up to a total volume of 50 µl, with the following final concentrations: 1x PCR buffer, 200 µM dNTPs, 0.5 µM of each primer and 2U DyNAzyme DNA polymerase (Finnzyme Oy, BioConcept). Primers used for the detection of homologous recombination were 5’-AAAACCTAAATTCTATGGGGCG-3’ (ko-7) and 5’-GGATGTGCTGCAAGGCG-3’ (lacZ.2). The primer ko-7 and 5’-GGAAGTGTCAATTGCCA-3’ (ko-6) were used to detect the endogenous Tl gene. Cycling conditions consisted of a single denaturing step at 94°C for 3 min, followed by a denaturing step at 94°C for 1 min, primer annealing at 60°C for 1 min and extension at 72°C for 2 min. 35 cycles were performed and the final extension was prolonged to 10 min. 10 µl of the PCR reaction was separated on a 1% agarose gel that was stained with SYBR-Gold (Molecular Probes) for 30-60 min thereafter. If homologous recombination
had occurred a 1.2 kb band was visible, whereas the size of the endogenous T1 is 1.1 kb. Using the above-described method, it was possible to detect a PCR product using 100 pKO3-transfected cells. It must be noted though that multiple copies of the test-targeting construct might have inserted into the chromosomal DNA which could have resulted in a higher apparent sensitivity. Therefore, it must be assumed that the DNA of more than 100 ES cells transfected with pKO4 are needed in order to detect homologous recombination.

**Karyotype analysis of ES cells** (140)
ES cells were incubated for 4 h in culture medium containing 0.06 µg/ml colcemid to arrest the cell cycle at its metaphase. The supernatant was collected and set aside. Cells were washed with PBS, trypsinised, the supernatant was readded and this cell-suspension was centrifuged for 5 min at 1000 rpm. After discarding the supernatant, the cell pellet was carefully resuspended in 3 ml of 1% KCl and incubated for 5 min at rt. Incubation in this hypertonic solution leads to swelling of the cells. The cells were centrifuged for 5 min at 500 rpm and resuspended in 5 ml fixative (MeOH : CH₃COOH = 3 : 1, prepared freshly and kept on ice). After at least 10 min, the suspension was centrifuged as above and the pellet resolved in 2 ml fixative. To burst the cells, aliquots of the suspension were dropped from about 60 cm height on a microscope-slide. The chromosomes were stained with DAPI (1:1000 diluted in PBS) (Sigma) for 15 min in the dark and counted under the fluorescence microscope.

**Screening ES cell cultures for mycoplasma contamination**
ES cells were grown in 6-well plates containing a sterile coverslip in each well. 500 µl of Carnoys fixation (EtOH : CH₃COOH = 3 : 1) were added dropwise to the ES cells without removing the supernatant and left for 5 min. Subsequently, the supernatant was removed and replaced with 500 µl of Carnoys fixation solution. After 10 min the solution was discarded and the cells air-dried. 2 ml of DAPI staining solution (as described above) was added to the cells for 10 min and then carefully removed and the cells were washed thoroughly with water. The coverslips were placed upside down on glass slides containing a drop of pre-warmed Mowiol (Hoechst). The cells were analyzed for extranuclear DNA staining under the fluorescence microscope.
Construction of the AAT-T1-Fc and the AAT-T1 transgene

The T1-FcIgGγ1 (T1-Fc) fragment was excised from the previously described plasmid pT1-Hyγ1 (30) with the restriction enzymes SacI and BamHI. After treatment with the Klenow fragment of DNA polymerase to generate blunt ends, it was subcloned into the EcoRV site of pBSKS(+) (Stratagene). Subsequently, the T1-Fc fragment was excised with XbaI and XhoI and ligated into the plasmid AAT-8, containing the human α-1 antitrypsin promoter (120), giving rise to the plasmid AAT-T1-Fc. To generate the AAT-T1 construct, the T1 ORF was PCR amplified from the T1 cDNA plasmid pT1.10 (1) with the upstream primer 5'-CGTCTAGACCACCATGATTGACAGACAGACAATGG-3' containing the Kozak sequence (underlined) and the downstream primer 5'-GCTCTAGATCATCAAGCAAT-GTGTGAGGGAC-3'. Both primers contain a 5' XbaI restriction site (marked in italics) for subcloning. The eukaryotic expression vector pSVK3 (Pharmacia) was linearized with XhoI and treated with Mung-bean nuclease (Pharmacia) to destroy the XhoI and the SacI sites in the poly linker. The linearized DNA was incubated with 2U of Mung-bean nuclease in 30 mM sodium acetate, pH 4.5, 50 mM NaCl, 1 mM ZnSO₄, 5% glycerol at 37°C for 10 min and religated thereafter. The XbaI–BamHI fragment of this plasmid, containing a SV40 splice and polyadenylation site, was excised and subcloned into the poly linker of pBSKS(+). The AAT promoter was inserted into the SacI/XbaI sites of this plasmid and subsequently, the T1 ORF XbaI fragment was cloned downstream of the AAT promoter fragment into the XbaI site, giving rise to the transgene construct AAT-T1. In order to be able to excise multiple copies of the transgene after insertion into chromosomal DNA, a lox sequence, which allows cre-mediated recombination, was cloned 5’ of the T1 sequence. Two oligos, 5’-ATAACTTCGTATAATGTATGCTATACGAAGTTATAGC-3’ and 5’-ATAACTTCGTATAGCATACATTATACGAAGTTATAGC-3’ that contain the lox sequence and a linker (in italics), which allows ligation into the SacI site of the targeting vectors, were phosphorylated with T4 polynucleotide kinase (Promega) and annealed (50 pmol of each oligo in 20 mM Tris-HCl, pH 8. 2 mM MgCl₂, 50 mM NaCl) by incubation for 5 min at 70°C and subsequent slow cooling to rt. Both transgene constructs were linearized with SacI and ligated with the annealed lox-oligo using a molar ratio of vector to insert of 1:3. Since linker addition destroyed the SacI site upon ligation, the lack of the SacI site was indicative for the insertion of the lox-oligo. For microinjection, the AAT-T1-Fc and the AAT-T1 sequences were excised from the constructs using PvuII, separated by gel electrophoresis, purified with the Gel Extraction kit (QIAGEN) and resuspended in 10
mM Tris-HCl, pH 8.5 at 100 ng/µl. Each fragment was further diluted to a concentration of 2 ng/µl and injected into the pronucleus of fertilized oocytes, derived from the hybrid strain B6C3F1, according to standard methods (121). Transgenic animals were backcrossed to C57BL/6 mice. The animals used for experiments derived from the second, third, and fourth backcross with C57BL/6 mice.

**PCR and Southern blot analysis with mouse tail DNA**

DNA was extracted from mouse tails by digestion with proteinase K (500 µg/ml) in 50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, and 1% SDS at 55°C, followed by DNA precipitation with isopropanol. The DNA was resuspended in TE, pH 8.0 and 200-500 ng chromosomal DNA taken for PCR analysis. Alternatively, to obtain a crude tail lysate that is suitable for PCR, but not Southern blot analysis, tails were digested with proteinase K (100 µg/ml) in 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.5 % NP-40, and 0.5% Tween-20 in a total volume of 200 µl at 55°C. Proteinase K was inactivated at 95°C for 10 min and the samples were centrifuged for 5 min at 14'000 rpm. 1-2 µl of the lysate were taken for PCR analysis. Primers used for the detection of T1-deficient mice were 5'-AAAACTAAAATTCTATGATGGGCG-3' (T1-upstream; ko-7) and 5'-GGATGTGCTGCAAGGCG-3' (lacZ-downstream; lacZ.2). The downstream primer 5'-GGAAGTGTCAATTGCCA-3' (ko-6) was used to detect the endogenous T1 gene of the heterozygous and wild-type mice. PCR was performed with DyNAzyme DNA polymerase (Finnzyme Oy, BioConcept) according to the manufacturer’s protocol. Cycling conditions consisted of a single denaturing step at 94°C for 3 min, followed by a denaturing step at 94°C for 1 min, primer annealing at 60°C for 1 min and extension at 72°C for 1.5 min. 30 cycles were performed and the final extension was prolonged to 10 min. Primers for the detection of transgene insertion were 5'-GCTTAAATCGGACGAGG-3' (AAT-promoter-upstream) and 5'-CTGATCCACGTACTGTCG-3' (T1-downstream). Cycling conditions were used as described above with the exception of an annealing temperature of 52°C and an extension time of 1 min. For Southern blot analysis 5 µg of genomic DNA were digested with SacI (T1-deficient mouse) or HindIII (T1-transgenic mice) and the blots hybridized with a DIG-labeled 1.9 kb XbaI-HpaI fragment, spanning the region between -2.7 and -0.8 kb relative to the transcription initiation site at the proximal exon 1 and either a 2.2 kb HindIII or a 2.0 kb HindIII–EcoRI fragment for DNA derived from the AAT-T1-Fc and AAT-T1 transgenic mouse, respectively. Southern blot analyses were performed using DIG High Prime DNA.
labeling and Detection Kit according to provided protocols (Roche Molecular Biochemicals).

Expression of the soluble T1 and T1-Fc protein in HepG2 cells
To test whether the T1 gene is expressed under the liver-specific α-1 antitrypsin promoter, the human hepatoblastoma cell line HepG2 (141) was transfected with the constructs AAT-T1 and AAT-T1-Fc. Transfection was performed using the SuperFect Transfection Kit according to the manufacturer’s protocol (QIAGEN). After transfection the cells were maintained in serum-free Hybridoma medium (GibcoBRL, Life Technologies) in order to have no immunoglobulins in the culture supernatant. Supernatants were harvested 24 and 48 h after transfection and concentrated 20-fold using a centrifugal filter device (Ultrafree-15, Biomax-10, Millipore). NIH 3T3 fibroblasts were used as control cells for T1 protein production. Cells were incubated in DMEM supplemented with 0.5% FCS to arrest cell growth. After 24 h, the cells were stimulated to reenter the cell cycle by replacing the growth medium with fresh DMEM containing 10% FCS. This stimulation leads to accumulation of T1 protein in the cell supernatant which was replaced 4 h after induction with serum-free Hybridoma culture medium and harvested 2 h thereafter. Supernatants were stored at -70°C until assayed.

Immunoprecipitation and immuno blotting of T1
For immunoprecipitation, 30 µl of protein-G-Sepharose (Pharmacia) were incubated with 500 µl of the concentrated cell supernatants or 60 µl mouse serum at 4°C on a rotating wheel o/n. Serum and supernatants deriving from cells transfected with AAT-T1 or from control NIH 3T3 cells were incubated with 30 µl of protein-G-Sepharose that was previously coated with anti-T1 mAb DJ8 (3 µg/30 µl protein-G-Sepharose) (12) for 1 h at 4°C. Supernatants of cells transfected with AAT-T1-Fc were incubated with uncoated beads. After washing the resins three times with PBS, the beads were boiled in reducing Laemmli sample buffer and the eluted proteins were electrophoresed through a 10% SDS-polyacrylamid gel. Proteins were transferred onto a PVDF membrane (Millipore) using the Mini Trans-blot system (BioRad). The membrane was blocked for 1 h in 5% milk-TBST (100 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween-20) and then incubated with affinity purified rabbit anti-T1 polyclonal Ab (7) (1:50 in 5% milk-TBST) at 4°C o/n. The blot was washed three times in TBST and then incubated with a HRP-labeled goat anti-rabbit IgG antibody (1/7000) (Southern Biotechnology Associates, Inc.,
Birmingham, AL) for 1 h in 5% milk-TBST at rt. After washing as above the blot was developed using the ECL system (Amersham, LIFE SCIENCE).

**Expression of the soluble T1-Fc protein in transgenic mice**
Liver tissue was homogenized in 1 ml PBS, supplemented with 40 µl of a 25x cocktail of protease inhibitors (CompleteTM, Roche Molecular Biochemicals), using a sonicator and cell debris were removed by centrifugation (1000xg, 5 min, 4°C). The protein concentration was determined according to the Bradford method (142) using Bradford reagent (BioRad) and known concentrations of BSA as standard. 40 µg of total protein were separated on an 8% SDS-polyacrylamid gel by electrophoresis and transferred onto a PVDF membrane. Western blot analysis was performed as described above. The expression of the T1-Fc transgene in the serum was tested by immunoprecipitation (as described above) using protein-G-Sepharose coated with anti-T1 mAb DJ8 (3 µg/30 µl protein-G-Sepharose).

**ELISA assay for determining T1-Fc concentration in serum of transgenic mice**
The concentration of blood T1-Fc was estimated by ELISA. Plates were coated with anti-T1 mAb (DJ8) (10 µg/ml) (12) in coating buffer (0.1 M NaCO₃, pH 9.6, 50 µl/well) and incubated o/n at 4°C. Plates were washed three times with PBS-T (0.05% Tween-20 in PBS) and blocked with PBS/4% BSA for 2 h at rt. Mice were bled and the serum was added in serial 2-fold dilutions starting with a 1/100 dilution in PBS/0.1% BSA. After incubation for 2 h, the plates were washed six times in PBS-T and incubated with the detecting HRP-labeled goat anti-human IgG antibody (1/5000; Southern Biotechnologies Associates, Inc.) for 1.5 h at rt. Plates were washed thereafter, the ABTS substrate (Roche Molecular Biochemicals) added (0.2 mg/ml ABTS in 100 mM NaH₂PO₄, pH 4, and 1 µl/ml 30% H₂O₂, added before use) and incubated until the color reaction developed. Absorbance was read at 405 nm in a Microplate Reader (BioRad Laboratories, CA). The standard curve was established using serial dilutions (10 µg/ml to 56 ng/ml) of the T1-IgG recombinant protein (12) and serum antibody concentrations were determined using Microplate Manager III software (BioRad Laboratories, CA). This standard ELISA protocol was used throughout this work. However, minor modifications such as different capturing and detecting antibodies were used for other experiments. These will be pointed out in the following sections of Materials and Methods.
Isolation, cultivation and cytofluorimetric analysis of bone marrow derived mast cells

Bone marrow (BM) cells were prepared by flushing femurs and tibias of mice with balanced salt solution (BSS) and cultured in 10% complete IMDM (see above) supplemented with 2% conditioned culture supernatant from IL-3-secreting X63/IL-3 cells (139). BM cultures were enriched for mast cells by repetitively transferring the suspension cell fraction into fresh culture flask every fifth day. After approximately four weeks in culture, > 90% of the cells displayed a typical mast cell-like phenotype, as shown by double-positive flow cytometric staining for c-kit and surface IgE receptor. Briefly, BM-derived cultured mast cells (5 x 10^5 cells) were incubated for 10 min with the 2.4G2 antibody (Fc Block, PharMingen) to block FcγII/III receptors, washed and incubated for 30 min at 4°C in FACS buffer (PBS containing 2% FCS and 5 mM EDTA) containing the appropriate combination of PE-conjugated anti-c-kit Ab (Caltag Laboratories), FITC-conjugated or biotinylated IgE (both obtained from K. McCoy) or FITC-labeled anti-T1 Ab (DJ8) (12). Bound biotinylated IgE was detected with PE-conjugated streptavidin (Caltag Laboratories) in a second staining step. After washing with FACS buffer, samples were analyzed using a FACScan flow cytometer and CellQuest software (Becton Dickinson, Mountain View, CA). To control for nonspecific surface staining, cells were stained with PE- and FITC-conjugated isotype matched antibodies (PE-conjugated rat IgG2b, c-kit isotype control, Caltag Laboratories, FITC-conjugated IgG1, T1 isotype control, BD PharMingen).

Detection of β-galactosidase by immunohistochemistry

Immunohistochemical analyses were performed with T1-deficient and wild-type embryos. They were taken on day 10, 12, 14, 16, 18 and 20 of embryonic development and snap frozen in liquid N2. 5 μm thick cryostat sections were cut and fixed for 10 min in aceton. Air-dried slides were washed three times in TBS (137 mM NaCl, 25 mM Tris-base, 2.7 mM KCl, pH 7.4) and blocked with TBS/1% FCS for 10 min. Sections were incubated with rabbit anti-β-galactosidase antiserum (1:800) (Cappel, Organon Teknika, West Chester, PA) in TBS for 60 min and washed three times in TBS. An alkaline phosphatase (AP)-labeled goat anti-rabbit Ig (1:80) (Jackson Immuno Research Laboratories, Inc. West Grove, PA) was added in TBS containing 5% normal mouse serum and incubated for 40 min, followed by incubation with a AP-labeled donkey anti-goat Ig (1:80 in TBS/5% normal mouse serum, 40 min) (Jackson Immuno Research
Laboratories, Inc. West Grove, PA). AP was visualized with naphtol AS-BI phosphate and new fuchsin yielding a red reaction product (DAKO New Fuchsin Substrate System, DAKO CORPORATION, Carpintera, CA). Endogeneous alkaline phosphatase activity was inhibited by the addition of 1 mM levamisole (Sigma). Sections were counterstained for 3 min with Mayer’s hemalum. Cytospins of T1-deficient and wild-type mast cells (1.2 x 10^6 cells/slide, 1000 rpm, 8 min) were used as controls.

**Polarization of transgenic CD4+ T cells into Th1 and Th2 cells**

Effector Th1 and Th2 cells were generated by *in vitro* polarization of naive transgenic tg7 CD4+ cells (116). Spleens of tg7 mice were removed and smashed through a grid to obtain a single-cell suspension. After a quick-spin at 1000 rpm to remove large cell aggregates, they were centrifuged for 5 min at 1400 rpm. The supernatant was discarded and the cells incubated with 20 µl CD4(L3T4) Microbeads (Miltenyi Biotec) in 300 µl MACS buffer/spleen (PBS containing 2% FCS and 5 mM EDTA) for 20 min on ice. The cells were washed, resuspended in 500 µl MACS buffer, passed through a cell strainer with a mash size of 70 µM (Falcon) and loaded onto a MACS separation column (Positive selection columns type VS+, Miltenyi Biotec) which had previously been equilibrated with 9 ml MACS buffer and was placed in the magnetic field of the MACS (magnetic activated cell sorter, Miltenyi Biotec). After the suspension had passed through the column, the column was washed with 9 ml MACS buffer. To recover the bound cells the column was removed from the magnetic field and the cells were eluted by applying 4 ml MACS buffer to the reservoir of the column and flushing out the cell suspension using the plunger supplied with the column. Cells were centrifuged as above, resuspended in 5 ml 10% complete RPMI, counted and adjusted to a concentration of 5 x 10^6 cells/ml. Aliquots of 5 x 10^5 cells were cultured in 24-well tissue culture plates in 2 ml 10% complete RPMI in the presence of 5 x 10^6 irradiated C57BL/6 spleen cells plus VSV-antigenic peptide p8 (VSV-G amino acids 415-433, SSKAQVFEHPH1QDAASQL, 1 µg/ml). Briefly, a single-cell suspension of C57BL/6 spleen was prepared as described above, adjusted to 5 x 10^7 cells/ml and irradiated at ~2000 rad (50 sec). 5 x 10^7 cells were incubated with 20 µg activating peptide p8 for 30 min at 37°C on a rocking platform. Th1 cultures were supplemented with 50 U/ml recombinant murine IL-2 (rmIL-2, PharMingen), whereas Th2 cultures contained rmIL-2 plus rmIL-4 (500 U/ml, PharMingen) and rat anti-mouse IFN-γ (1:100 dilution of hybridoma supernatant, AN-18.17.24, (125)). One spleen yields approximately 5 x 10^7 cells, of which about 25% are
CD4+ T cells. After 4 days in culture at 37°C, cells were washed and expanded for a further 5-6 days in 6-well tissue culture plates with 5 ml 10% complete RPMI containing rmIL-2 (Th1 cultures) or rmIL-2 plus rmIL-4 and anti IFN-γ (Th2 cultures). For the second stimulation, Th1 and Th2 cells were harvested, centrifuged and resuspended in culture medium to obtain a concentration of 5 x 10⁶ cells/ml. Irradiated C57BL/6 spleen cells were prepared as described above, loaded with p8 and incubated with Th1 and Th2 cells. After 3-4 days in culture, cells were expanded as before and grown for 5-6 days before the next round of stimulation. After three rounds of in vitro stimulation irreversibly polarised effector populations were obtained (117, 143). Cytofluorimetric analyses of intracellular cytokines to determine the Th1 and Th2 phenotype were performed using aliquots of 5 x 10⁵ CD4+ T cells that were stimulated in vitro at 37°C for 4 h in 10% complete RPMI containing PMA (50 ng/ml), ionomycin (500 ng/ml) and monensin (2 μM, all from Sigma). Samples were stained as described in details below.

T cell proliferation assay

Polystyrene microspheres, 5 μm in diameter (Interfacial Dynamics Corporation, Portland, OR) were coated with antibody by incubation with both 10 μg/ml anti-CD3 mAb (2C11) and 5, 10, 15 or 20 μg/ml of either anti-Tc mAb (DJ8) or control IgG1 ab (F389, L. Hangartner). The beads were coated at a concentration of 10⁷ beads/ml in PBS and incubated for 2 h at 37°C on a rotating wheel, and subsequently washed three times with PBS. Beads were incubated in 1 ml IMDM containing 5% FCS for 30 min prior to the addition to the T cell cultures in order to block nonspecific binding. The beads with different antibody concentrations were added at 10⁷/well to in vitro-polarized Th1 and Th2 cells that were cultured at 10⁵ cells per well in 96-well round-bottom tissue culture plates. Each sample was prepared in triplicates. The cells were grown in 10% complete IMDM supplemented with soluble anti-CD28 mAb (1:10 final dilution of hybridoma supernatant). Proliferation was quantitated after 24, 48 or 72 h in culture by adding of 1 μCi [³H]thymidine (Amersham,GB) per well and measuring [³H]thymidine incorporation after 8 h using a beta counter (Wallac, Turku, Finland). The same experimental set up was used for the analysis of cytokine production. After 60 h incubation, cell supernatants were collected and IL-4, IL-5, and IFN-γ production was measured by ELISA (see below).
**N. brasiliensis cultures**

*N. brasiliensis* was cultured by passage through 6-12 week old Lewis rats. These rats were inoculated s.c. with 4000–5000 infective third-stage larvae (L₃) in 0.5 ml dH₂O into the leg flank. Rat fecal pellets were collected between day 7 and 12 post infection and soaked in 50 ml dH₂O for 10 min. Fecal pellets were mixed with an equal volume of granular activated charcoal (Sigma) to form a paste and then spread out onto a moist filter paper that was underlayed with a wet absorbent cloth in a large petri dish. The fecal culture was incubated at 25°C for 5 days to 4 weeks. The exsheathed L₁ larvae migrate to the edge of the filter paper where they could be harvested by swirling water around the edge of the filter paper to dislodge the larvae.

**N. brasiliensis inoculation and quantitation of worms and eggs**

Eight to twelve-week old mice were inoculated with third-stage infective larvae (500-750 L₃) by s.e. injection in a total volume of 0.2 ml. Parasite egg numbers were determined from feces from individuals or groups of mice, starting at day 5-6 p.i. 25 fecal pellets were collected daily and soaked in 12.5 ml dH₂O for at least 30 min. After vigorous vortexing, an equal amount of saturated NaCl was added. The mixture was inverted several times and an aliquot was immediately removed and transferred to a McMaster Worm Egg Counting Chamber (Weber Scientific International Ltd.). The eggs floated to the surface and were counted within the grid. Since the counting chamber contains 150 µl, the number of counted eggs was divided by 0.15 to give eggs/ml (eggs/fecal pellet), and this number was multiplied by 25 to yield eggs/gram feces (25 mouse fecal pellets = 1 gram).

Adult worm numbers were determined by removing the small intestine, slicing it open longitudinally, cutting it into small pieces and placing the pieces on a gauze bag that was submerged in PBS at 37°C for at least 60 min. Most worms migrated through the gauze to the bottom of the tube and could be collected and counted. The small intestine was removed from the gauze, spread between two glass plates and analyzed microscopically for any remaining worms.

**Determination of peripheral blood and tissue eosinophilia**

Blood smears were collected every three days p.i. and stained with Diff-Quik® (DADE BEHRING AG, Düdingen, Switzerland). The number of eosinophils, neutrophils, lymphocytes and macrophages in 200 cells were counted based on morphology and
staining characteristics and the percentage of eosinophils calculated. For determination of lung eosinophil infiltration, a bronchoalveolar lavage (BAL) was performed by making a small cut across the exposed trachea of anaesthetized mice and the lungs were washed with 1 ml of PBS. A volume of approximately 800 µl was removed and put into weighed Eppendorf tubes containing 50 µl FCS. The tubes were then re-weighed to determine the exact amount of BAL fluid obtained. BAL fluid was centrifuged gently to pellet the cells and the supernatant removed leaving approximately 120 µl. Viable BAL cells were counted using trypan blue dye exclusion (0.4% Trypan Blue Stain, GibcoBRL, Life Technologies). Cells were resuspended in a volume of PBS to give a final concentration of 1.0 x 10^6 cells/ml or in a minimum volume of 100 µl. BAL cells were used to prepare cytospins (100 µl, 1000 rpm, 8 min) which were stained with Diff-Quik®. A minimum of 200 cells was counted and the percent eosinophils and total eosinophil number/ml of BAL cells calculated.

Isolation of lymphocytes from lymph nodes or spleen
Spleen and lymph nodes (mediastinal, mesenteric and inguinal LN) were removed from mice and smashed through a grid with a plunger to obtain a single-cell suspension. Cell aggregates were removed by a quick-spin, the supernatant transferred to a new tube and centrifuged for 5 min at 1200 rpm. The cell pellet was resuspended in 10% complete IMDM and the cell number determined.

Isolation of lung lymphocytes
Lungs from N. brasiliensis infected mice were flushed with PBS through the vena cava, removed, cut into small pieces and incubated for 90 min at 37°C in BSS supplemented with 1 mg/ml DNase (Roche Molecular Biochemicals) and 2 mg/ml Collagenase D (Roche Molecular Biochemicals). Single-cell suspensions were obtained by shearing the tissue through an 18 G needle. After a quick-spin, cells were centrifuged for 5 min at 1000 rpm and resuspended in 5 ml BSS. Lymphocytes were separated through a Ficoll gradient (density 1.077 g/liter, Biochrom) by underlying 5 ml cell suspension with 5 ml Ficoll followed by centrifugation at 2000 rpm for 10 min. The lymphocyte-containing interface was removed with a pipette, washed once in BSS and resuspended in 10% complete IMDM.
Cytofluorimetric analysis of intracellular cytokines

2-5 x 10^5 lymphocytes isolated from lymph nodes or lungs were restimulated for 4 h on anti-CD3 mAb-coated plates (10 µg/ml, 2C11) and in presence of IL-2. Spleen cells or CD4+ T cells in culture were restimulated for 4 h with PMA/ionomycin (50 ng/ml PMA and 500 ng/ml ionomycin, both from Sigma). All cells were incubated in 10% complete IMDM supplemented with monensin (2 µM, Sigma). Surface expression of T1 and CD4 as well as intracellular production of various cytokines such as IL-4, IL-5, IL-10, IL-13, TNF-α, and IFN-γ was determined by immunofluorescence staining and flow cytometry using the following antibodies: anti-CD4-Tri-Color (Caltag Laboratories), anti-T1-FITC (DJ8), anti-IL-4-PE, anti-IL-5-PE, anti-IL-10-FITC, anti-IFN-γ-PE/FITC, and anti-TNF-α-FITC (all from BD PharMingen). Restimulated cells were harvested and washed in 2 ml FACS buffer (PBS containing 2% FCS and 5 mM EDTA). Samples were stained for 30 min at 4°C with anti-CD4-Tri-Color and/or anti-T1-FITC antibodies. After washing, cells were fixed by incubation in 100 µl of PBS/4% paraformaldehyde for a minimum of 10 min at 4°C. In some experiments the cells were stored in this solution o/n. For intracellular staining, the cells were permeabilized by the addition of 2 ml permeabilization buffer (PB: PBS/0.1% (w/v) saponin/ 0.05% sodium azide, both from Sigma) for 10 min. Samples were stained for 30 min at 4°C in PB containing the appropriate combination of anti-cytokine antibodies. After washing twice with PB, samples were resuspended in 500 µl FACS buffer and analyzed using a FACScan flow cytometer and CellQuest software (Becton Dickinson, Mountain View, CA). To control for nonspecific intracellular staining, parallel samples of stimulated and permeabilized cells were stained with PE- or FITC-conjugated isotype matched mAbs of irrelevant specificity. Unstimulated control cells were also included in the staining procedures.

Determination of IL-4 and IL-5 secreting cells by ELISPOT

96-well plates (Petra plastic) were coated with anti-CD3 mAb (10 µg/ml, 2C11) and either rat anti-mouse IL-5 mAb (10 µg/ml, TRFK-5, PharMingen) or rat anti-mouse IL-4 (10 µg/ml, 11B11, (124)) in coating buffer (0.1 M NaCO₃, pH 9.6, 50 µl/well) and incubated o/n at 4°C. The plates were washed three times with BSS, blocked with PBS/5% BSA for 30-60 min, washed again as before and then incubated with 10% complete IMDM for 10 min. Lymphocytes isolated from the lungs of infected mice were prepared as described above and restimulated on the Ab-coated plates for 10 h in 10% complete IMDM and IL-2. The cells were added serially diluted (2-fold), starting from 5
x 10⁵ cells/well in a volume of 200 μl/well. Cells were then removed by washing for six
times with PBS-T (0.05% Tween-20 in PBS) and one time with dH₂O to lyse any
remaining cells. The detecting antibodies, rat anti-mouse IL-5 (5 μg/ml, TRFK-4-biotin,
(123)) or rat anti-mouse IL-4 (1 μg/ml, anti-IL-4-biotin, PharMingen), were added in
PBS/0.1% BSA for 2 h at rt. The plates were washed as above, omitting washing with
dH₂O, and incubated with alkaline phosphatase-conjugated streptavidin (1/1000 in
PBS/0.1% BSA, Promega) for 1 h at rt. The washing procedure was repeated and 150
μl/well of the substrate 5’BCIP (1 mg/ml) in Alkaline buffer solution (Sigma) was added
and incubated until spots developed. The substrate was flicked out, the plate air-dried and
the amount of spots enumerated.

**Determination of cytokines and serum antibody by ELISA**

Lymphocytes isolated from the lungs or lymph nodes of infected mice were prepared as
described above and restimulated with immobilized anti-CD3 mAb (10 μg/ml, 2C11) and
IL-2. Supernatants were removed after 60 h in culture and stored at -20°C until assayed.
For antibody determination mice were bled from the retro-orbital plexus every sixth day
and total serum IgE and IgG1 levels were determined by ELISA (as described in detail
above). Briefly, polyvinyl chloride 96-well plates were coated with 5 μg/ml rat anti-
mouse IgE (R35-72, PharMingen), 2.5 μg/ml rabbit anti-mouse IgG (Zymed, S. San
Francisco, CA), 2.5 μg/ml rat anti-mouse IL-5 (TRFK-5), 2.0 μg/ml rat anti-mouse IL-4
(11B11, (124)), 5.0 μg/ml rat anti-mouse IFN-γ (AN-18.17.24, (125)) or 3.0 μg/ml rat
anti-mouse IL-13 (clone 38213.11, R&D Systems) mAbs. Plates were blocked with
PBS/4% BSA for 2 h. Dilutions of serum or culture supernatants and standards were
added in PBS/0.1% BSA, incubated for 2 h at rt and bound immunoglobulin or cytokine
was detected with 2.5 μg/ml rat anti-mouse IgE (R35-118-biotin, PharMingen), 1 μg/ml
rabbit anti-mouse IgG1-HRP (Zymed), 0.5 μg/ml rat anti-mouse IL-4 (BVD6-24G2-
biotin, PharMingen), 5.0 μg/ml rat anti-mouse IL-5 (TRFK-4-biotin, (123)), 2.0 μg/ml
rat anti-mouse IFN-γ (XMG1.2-biotin, PharMingen) or 0.2 μg/ml biotinylated rat anti-
mouse IL-13 (R&D Systems) followed by incubation with peroxidase-conjugated
streptavidin (Jackson Immuno Research Laboratories, Inc. West Grove, PA) for biotin-
labeled antibodies. The reaction was developed with ABTS and the OD was read at 405
nm in a Microplate Reader. Cytokine or total serum antibody concentrations were
determined using Microplate Manager III software (BioRad Laboratories, CA) and were
calculated using IgE mAb (anti-TNP, clone TIB-141, ATCC), IgG1 mAb (Zymed) or recombinant cytokines (PharMingen, R&D) as standards.

**Infection with vesicular stomatitis virus (VSV)**
Mice were immunized intravenously with $2 \times 10^6$ pfu of live VSV serotype Indiana (VSV-IND). Sera were collected by bleeding from the retro-orbital plexus at different time points after immunization (day 4, 8, 12 and 20) for the determination of VSV-specific neutralizing antibody titers using a plaque assay.

**Serum neutralization test**
Vero cells were trypsinised and adjusted to a concentration of $1.5 \times 10^5$/ml in 5% complete MEM (a confluent T75 flask should provide enough cells for ten 96-well plates). Aliquots of 100 µl (1.5 x 10^5 cells) were added to each well of 96-well flat bottom plates, which were incubated o/n at 37°C. Sera were treated in two different ways: the reduced samples, that were used to measure IgG only, were prepared by incubating 20 µl serum with 20 µl of 0.1 M 2-ME in saline for 1 h at rt. Then 760 µl of 5% complete MEM were added to each sample. The non-reduced samples that were used to measure total neutralizing antibodies were directly diluted 40-fold in 5% complete MEM (20 µl serum and 780 µl medium). All samples were heat-inactivated for 30 min at 56°C. Serial 2-fold dilutions of sera in 5% complete MEM (100 µl) were mixed with equal volumes of VSV diluted to contain 500 pfu/ml. A control sample without serum and one with a neutralizing anti-VSV antibody was included. The mixture was incubated for 90 min at 37°C for virus neutralization. 100 µl of the serum-virus mixture was then transferred onto Vero cell monolayers in 96-well plates and incubated for 1.5 h at 37°C. Monolayers were overlaid with 100 µl of DMEM containing 1% methylcellulose. After incubation for 24 h at 37°C the overlay was flicked off, the monolayer was fixed and stained with 0.5% crystal violet for 1 h and washed thoroughly thereafter. The highest dilution of serum that reduced the number of plaques by 50% was taken as the titer.

**Detection of VSV specific antibodies by ELISA**
Since a detailed ELISA protocol has been described earlier, only the VSV-specific reagents will be mentioned here.
To test VSV-specific antibodies of subtypes IgG1, IgG2a, and IgG2b in the sera of infected mice, 96-well plates were coated overnight at 4°C with 10 µg/ml PEG-
precipitated VSV-IND resuspended in coating buffer. (Due to technical problems plates were coated with 1 μg/ml goat anti-mouse IgG1, anti-IgG2a, or anti-IgG2b [Southern Biotechnologies Associates, Inc.], that detect total IgG1, IgG2a and IgG2b instead of only VSV-specific IgG subtypes, in the assumption that an increase in a particular subtype after VSV-infection would represent mainly VSV-specific antibodies.) Sera were initially prediluted 40-fold in PBS/0.1% BSA and then serially diluted 2-fold. HRP-labeled specific rabbit anti-mouse IgG1, IgG2a, or IgG2b (1 μg/ml, Zymed) were used as detecting antibodies. As standards, purified myeloma IgG1, IgG2a, and IgG2b (Zymed) were used at 10 μg/ml, serially diluted 2-fold.

**Infection with Lymphocytic Choriomeningitis virus (LCMV)**

Mice were infected with 200–300 pfu LCMV-WE in a total volume of 20–30 μl into the right and left hind footpads. Footpad swelling was monitored daily, starting on day 6 after infection until the swelling reaction declined (approx. day 12, day 0 was included as reference), using calipers (Kroeplin). Blood was taken on days 0, 8, 12, 15, and 20 p.i. to measure the LCMV nucleoprotein-specific antibodies in the serum and on day 58 p.i. to measure the neutralizing antibodies (132).

**LCMV nucleoprotein-specific ELISA**

A detailed ELISA protocol has been described earlier, only the LCMV-NP-specific reagents are listed here.

96-well plates were coated with recombinant LCMV nucleoprotein (5 μg/ml), that had previously been sonicated for about 20 sec. in coating buffer and incubated o/n at 4°C. After blocking, the serum was added with a starting dilution of 1/30 and then diluted 3-fold across the plate. Bound IgG was detected with 1 μg/ml HRP-conjugated goat anti-mouse IgG (γ chain-specific, Sigma) followed by incubation with the ABTS substrate.

**Culturing Listeria monocytogenes for injection**

A fresh culture was made from a frozen stock (-70°C) in 10 ml Trypticase Soy Broth (BBL Microbiology Systems) and grown o/n on a shaker at 37°C. The o/n culture was diluted 1:10 with fresh medium and incubated again for 4–5 h. The Listeria titer was estimated by spectrophotometry (1 OD600 = 109 cfu/ml) and the culture was diluted to a concentration of 104/ml. The dose used to immunize mice is 2000–5000 cfu given i.v. in 200 μl PBS. Because measuring OD gives only an estimate of cfu/ml, 0.1 ml of 10-fold
dilutions of the culture to be injected were plated on agar plates made of Tryptose Blood agar (Difco) and counted the next day.

**Determining organ titers**

Listeria titers were determined in the spleen and in the liver at day 3 and 5 after infection. Organs were removed aseptically and kept in BSS on ice. Subsequently, they were homogenized and diluted in 4 steps of 10-fold dilutions. The dilutions were made in BSS and 10 μl of every dilution was plated on 1/4 agar plates (Tryptose Blood Agar). The plates were incubated o/n at 37°C and on the following day the number of colonies was determined.
7. Abbreviations

2-ME 2-mercaptoethanol  
AAT α-1-antitrypsin  
Ab antibody  
AP alkaline phosphatase  
AP-1 activator protein 1  
APC antigen presenting cell  
BAL bronchoalveolar lavage  
BM bone marrow  
bp base pairs  
BSA bovine serum albumine  
BSS balanced salt solution  
CFU colony forming units  
CTL cytotoxic T cell  
DAPI 4′-6′-diamino-2-phenylindole  
DMEM Dulbecco’s modified Eagle’s medium  
DMSO dimethyl sulfoxide  
DNA deoxyribonucleic acid  
DTH delayed type hypersensitivity  
ELISA enzyme linked immunosorbent assay  
ES cells embryonic stem cells  
FACS fluorescence activated cell sorter  
FCS fetal calf serum  
FITC fluorescein isothiocyanate  
HRP horseradish peroxidase  
i.v. intravenously  
IFN interferon  
IL interleukin  
IL-1R interleukin-1 receptor  
IL-1ra interleukin-1 receptor antagonist  
IL-1RAcP interleukin-1 receptor accessory protein  
IL-1Rrp interleukin-1 receptor-related protein  
IMDM Iscove’s modified Dulbecco’s medium  
kilobase
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>kbp</td>
<td>kilobasepair</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>L. major</td>
<td>Leishmania major</td>
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<tr>
<td>L. monocytogenes</td>
<td>Listeria monocytogenes</td>
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<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LN</td>
<td>lymph nodes</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MACS</td>
<td>magnetic activated cell sorter</td>
</tr>
<tr>
<td>MAP kinase</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEFs</td>
<td>mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>N. brasiliensis (Nb)</td>
<td>Nippostrongylus brasiliensis</td>
</tr>
<tr>
<td>Neo</td>
<td>neomycin</td>
</tr>
<tr>
<td>NFκB itogen</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NP</td>
<td>nucleoprotein</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>p.i.</td>
<td>post infection</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PGK</td>
<td>phosphoglycerate kinase</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transkriptase PCR</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS polyacrylamid gel electrophoresis</td>
</tr>
<tr>
<td>T1-M</td>
<td>membrane anchored form of T1 protein</td>
</tr>
<tr>
<td>T1-S</td>
<td>secreted soluble form of T1 protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>Th1 cell</td>
<td>T helper cell type 1</td>
</tr>
<tr>
<td>Th2 cell</td>
<td>T helper cell type 2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>TNF-β</td>
<td>tumor necrosis factor β</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TRE</td>
<td>TPA-responsive element</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
<tr>
<td>wt</td>
<td>wild-type</td>
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8. References


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Publications


Poster presentations

Senn, K., McCoy, K., Klemenz, R. T1-deficient and T1-Fc transgenic mice develop a normal protective Th2-type immune response following infection with *Nippostrongylus brasiliensis*. Annual assembly of Swiss Society for allergology and immunology, April 2000, Basel, Switzerland.

Senn, K., McCoy, K., Klemenz, R. T1-deficient and T1-Fc transgenic mice develop a normal protective Th2-type immune response following infection with *Nippostrongylus brasiliensis*. Keystone Symposium, Cytokines and Disease, April 2000, Utah, USA.
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