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HABILITATIONSSCHRIFT

"Identification and Characterization of a Novel Membrane Protein T cell immune response cDNA 7 (TIRC7) as a target for immunomodulation"

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1. CURRENT THERAPEUTIC UNMET NEED OF THE UNDESIRED IMMUNE ACTIVATION

Undesired immune response causes several disorders, such as organ rejection after transplantation and autoimmune diseases, including Rheumatoid Arthritis (RA) and Multiple Sclerosis (MS)[1,2].

Transplantation is an established therapy for patients with end-stage organ failure. The introduction of calcineurin inhibitors (CNIs) has remarkably improved the outcome of transplantation; however they are associated with significant life-threatening side effects[2-4]. Life-long administration is usually required to retain transplanted organs, but opportunistic infection and malignant tumors may develop as a consequence of the chronic nonselective immunosuppression[4,5]. Numerous studies have been attempted to reduce the dose and accordingly decrease the side effects of CNIs, especially through combination with other compounds[2,6-10].

Recently, experimental manipulation of regulatory signals during the immune response by antibodies or soluble proteins targeting molecules expressed on lymphocytes has shown some beneficial therapeutic effect suggesting a potential to establish tolerance by inducing anergy in activated, alloreactive lymphocytes[6,10,11].

The causes of a number of autoimmune diseases, such as RA are not completely understood, but a variety of cellular and molecular changes like leukocyte recruitment, inflammatory cytokine production, synovial tissue activation, hyperplasia and pannus formation, and osteoclast mediated bone erosions have been observed in the affected tissues[12]. RA is treated with a combination of medications, such as disease modifying antirheumatic drugs (DMARDS) that are able to retard disease progression over time. However, there is currently no cure for RA and the current therapeutic approaches are largely palliative. Medications to reduce symptoms of pain and swelling include NSAIDs (non-steroidal anti-inflammatory drugs), analgesics and corticosteroids. These drugs have little or no effect on disease progression or permanent joint damage. The new biologic-response modifiers, such as Enbrel (which specifically targets TNF) have been

highly successful new drugs[13,14]. Yet, these treatments are still only partially effective (i.e., palliative) as assessed in clinical trials employing standard efficacy measures. Thus, there exists substantial need for development of new RA therapies.

1.1. Activation of the immune system

Recognition of an antigen is initiated via the interaction between the recipient T cell receptors (TCR) and major histocompatibility complex (MHC) molecules[16-20]. MHC molecules present antigenic peptides to lymphocytes. There are two types of MHC molecules: class I and class II. Class I is expressed on virtually all nucleated cells and presents peptides derived from intracellular proteins to CD8 positive T cells. Class II is expressed on a subset of cells, such as dendritic cells (DC), macrophages, B cells, and activated T cells. These cells internalize, process and present foreign antigens to CD4+ T cells. Cells that efficiently process and present antigenic peptides to T cells are collectively called antigen presenting cells (APC). Activation of T cells is regulated by other molecules and cytokines in addition to the interaction between MHC and TCR. T cell activation also depends on the amount and types of antigen, and strength of binding[16,20,21]. Some molecules deliver negative signals and inhibit T cell activation, whereas other molecules are required for adhesion and stabilization of cellular interaction promoting stimulation[15,16,20-23]. The first signal to initiate T cell activation (signal 1) is generated by the interaction between MHC and peptide expressed on APC and TCR on recipient T cell. In addition to this first signal, supplementary signals from interaction of co-stimulatory molecules with their ligands are required for full T cell activation. Co-stimulatory signals are provided by receptor ligand pairs, such as TNF/TNFR and CD28/B7[16,22]. A blockade of the second signal can cause inhibition of proliferation and induction of apoptosis or anergy[6,16,23,24]. Once all essential activation signals are delivered to the T cell, an intracellular signaling cascade, which includes activation of nuclear transcription factors, such as NFAT and AP-1, causes the T cell to

enter the proliferation and differentiation phase. Activated T cells produce the cytokine IL-2 and express its receptor, IL-2R, at the cell surface. IL-2 shows both autocrine and paracrine behavior and is essential for optimal proliferation and differentiation of T cells into effector cells. Several other cytokines are also required to activate T cells, forming a network to regulate T cell differentiation into different subsets[25].

1.2. Immunomodulatory therapeutic compounds currently in clinical use

Due to progress in genomic research and as significant new knowledge has been gained about signaling pathways, selective therapies with a number of possible "points of engagement" seems to be feasible from differentially expressed proteins and their ligand interactions. However, broad and unspecific immune suppression remains the standard therapy. New treatments are solely used as add on therapeutic opportunities, especially during progression of disease.

The treatment of inflammatory diseases, such as RA involves as baseline therapy methotrexate, corsticosteroids and NSAIDS, including the COX-2 inhibitors. The recently established tumor necrosis factor (TNF) blockade through biologics (etanercept, infliximab)[13,14] is regarded as major therapeutic improvement. Therefore, the impetus to clinically test new biologics compounds significantly increased. However, the frequent return of disease after termination of therapy and the failure of TNF-blockade in a significant number of patients indicate the need for therapies that will modify other key signaling pathways of immune response to autoantigens.

New approaches targeting CD3, CD4, CD52, IL-1 and IL-6 resulted in some beneficial effects. As the role of B cells in inflammatory diseases became prominent, several recent clinical trials utilizing CD20 molecule have focused on B cell depletion.

In addition, CTLA4-Ig which modulates T cell response has shown promising results in the treatment of RA, as an alternative to TNF-alpha blockade[26].

The therapeutic landscape in transplantation involves several immunosuppressive agents, which exerts their effect on various stages of the T cell signaling cascade[27]. Initial immunosuppression therapy to prevent organ rejection usually employs a combination of several agents. CNIs are pivotal drugs to suppress immune response. However, because of their toxicity, they are usually utilized in combination with other compounds to reduce the dose and side effects[2,7-9]. Corticosteroids inhibit pro-inflammatory cytokines (IL-1, IL-2, and TNF-alpha) whereas calcineurin inhibitors (Cyclosporin A or FK506) suppress IL-2 production. Azathioprine and mycophenolate mofetil inhibit purine synthesis. Rapamycin blocks intracellular effects of IL-2[27,28,29].

Biological agents used to prevent rejection are widely used as therapy in the treatment of acute rejection episodes[30]. Antithymocyte globulin (ATG) is a polyclonal rabbit anti-human antibody that acts via depletion of peripheral blood lymphocytes by complement-dependent lysis and apoptosis induction, resulting in strong immunosuppression[31,32]. Repetitive administration is limited by the production of human anti-rabbit immunoglobulin, which neutralize ATG and can cause serum sickness[31,32].

OKT3, a monoclonal antibody against the CD3 component of the TCR complex, suppresses signal 1 and the formation of neutralizing human antibody against OKT3 is reported in almost 40% of patients who receive the monoclonal antibody[33,34,35]. Antibodies against the IL-2 receptor block IL-2 dependent signaling and are currently in clinical use in combination with other immunosuppressive drugs[27,30]. Monoclonal antibodies against the IL-2 receptor (basiliximab and daclizumab) have been used for induction therapy and show effectiveness in preventing acute rejection without marked adverse effects[36].

Due to the combined use of several immunosuppressive agents after transplantation the acute rejection episodes after one year of transplantation has been considerably reduced, however, chronic rejection of transplants remains a significant problem.

Moreover, the difficulty to detect rejection episodes at early time points, before organ injury, in order to be able to adjust the therapeutic treatment regime is a significant further challenge in the transplantation field. The prevention of transplant rejection is currently achieved by the continuous treatment with CNIs, which is also associated with significant toxic effects. A monotherapy with currently available antibodies is not possible due to their lack of efficacy as monotherapy.

In summary, due to the non-responsiveness or side effects of current treatments of transplanted organ rejection and autoimmune diseases there is still a high unmet need for drugs with better specificity and efficacy.

2. MATERIAL AND METHODS - AIM OF THE STUDY

The aim of our study was to identify and characterize novel genes that are differentially expressed in human immune cells early after allostimulation. Identification of such target molecules should lead to the discovery of new therapeutic interventions for the interruption of early lymphocyte activation to treat undesired immune response.

2.1. EXPERIMENTAL DESIGN AND METHODS

2.1. a. DDRT- PCR analysis of human peripheral blood mononuclear cells

Differential display reverse transcription – PCR (DDRT-PCR) analysis[36] enables a semiquantitative comparison of thousands of mRNA species from control and alloactivated cells simultaneously[37,38]. Because the methodology does not depend on the use of probes that are homologous to previously identified "activation genes", our experimental design was weighted towards the discovery of novel genes associated with human immune cell activation.

We have utilized a modification of the original technique[37,38,39] for DDRT-PCR, which permits us to target internal coding sequence of mRNA's instead of 3' untranslated regions. This has allowed us to define possible functions of upregulated messages based

on structural homology to well defined proteins in the database. Northern blot analyses using these coding region cDNA fragments as probes show that >90% of these mRNA's are regulated in a manner consistent with the DDRT-PCR results. In collaboration with Dr. Steven Gullan's laboratory, we have successfully used this method to analyse the expression of thousands of cDNA`s (i.e., mRNA's) and have identified and cloned several upregulated cDNA`s from human lymphocytes.

First, we used DDRT-PCR to analyse mRNA expression in resting peripheral monocytes of 8 normal individuals of diverse genetic backgrounds. After quantitatively analysing 1,000 cDNA`s we found that greater than 97% of the cDNA fragments are expressed at comparable levels in all individuals studied. Thus, changes in basal gene expression are unlikely to be a significant problem in the proposed study.

2.1. b. DDRT-PCR analysis of stimulated human peripheral blood lymphocytes

For alloantigen stimulation, the mixed lymphocyte culture as a model of T cell activation was used. mRNA changes in responder T cells were assessed before and at early sequential time points after exposure to alloantigen (irradiated allogenic lymphocytes).

Human T cell clones reactive to human myelin basic protein (MBP) epitope between 85-99 amino acids were activated with mitogen (PHA) and total RNA was isolated after 48 h of activation.

DDRT-PCR analysis was performed from allo- or MBP-activated and non-activated human lymphocytes for gene expression studies. Upregulated cDNA`s were isolated, cloned, sequenced and compared to the DNA and protein databases. Homology to known proteins in the database were used to determine whether specific cDNA`s are of interest because of functional motifs in the sequence. Northern analysis was used to confirm the differential gene expression.

2.2. Summary of the results obtained from DDRT-PCR analysis of human lymphocytes

We isolated cDNA`s from the *in vitro* studies on the basis of increased expression soon (<48 h) after activation. These cDNA fragments were sequenced and examined by northern analysis to confirm differential expression in human lymphocytes. From this group of differentially expressed genes we selected 6 novel cDNA`s (TGAM77[38], TGAP7 (GTPase homologue), TZON7 (mitochondrial carrier protein), TZAP7 (notchless protein), histone deacetylase (HDAC3)[39] and TIRC7[40] for further analysis, including cloning and functional characterization of full-length cDNA`s.

We selected these novel cDNA`s to clone based upon the following criteria: 1) unambiguous upregulation in northern analysis; 2) a reasonable size mRNA (< 7 kb); 3) early, transient changes in expression; 4) putative functional identification based on deduced amino acid homology to other known genes and confirmation of the biological functions of novel transciptionally regulated cDNA`s.

The TIRC7 molecule represents currently the most widely characterized target obtained from the study of DDRT-PCR of alloactivated lymphocytes. The following section of this scientific summary will focus on TIRC7 and its function in the immune response.

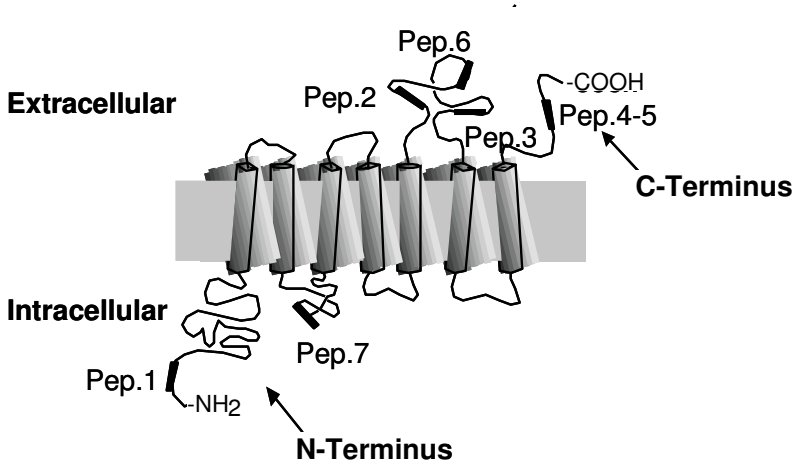
3. FUNCTIONAL CHARACTERIZATION OF THE TIRC7 PROTEIN

3.1. Molecular features of TIRC7

TIRC7 is a membrane protein, encoded as a 2488 base pair cDNA, which contains an open reading frame of 1842 nucleotides with a predicted protein length of 614 amino acids[40]. The prediction of the secondary structure reveals as a seven transmembrane (7-TM) protein with an intracellular N - terminus and extracellularly oriented C - domain (Fig.1). TIRC7 contains multiple putative sites of post-translational modification, including phosphorylation sites and N-linked glycosylation sites. No amino acid homology was found with any known proteins involved in T cell activation and it does contain an

immunoreceptor tyrosine-based inhibitory motif (ITIM), which indicates that it is involved in negative regulation of immune response.

Figure 1. Predicted TIRC7 molecular structure and peptide domains (Pep.1-Pep.6) used to generate antibodies.



It is of note that co-stimulatory molecules discovered to date all belong to either the immunoglobulin superfamily or the TNF-Receptor superfamily. In this regard, the predicted 7-TM structure of TIRC7 appears to be quite unique, in spite of its atypical extracellular C-terminus.

After activation of T cells by antigen, a high local concentration of a number of molecules required for T cell activation congregate at the site of membrane adhesion between T cell and APC, which is referred to as the immunological synapse. As described earlier, preformed TIRC7 proteins in the cytoplasm of resting T cells are rapidly transported to the cell surface on activation. Confocal and electron microscopic analysis demonstrated that the cell surface distribution of TIRC7 molecules is enriched towards antigen binding sites. Interestingly, when TIRC7 is expressed on the cell surface, it co-localizes with the T cell receptor (TCR)/CD3 complex indicating its participation in the T cell immune synapse[41](Figure 2A and B).

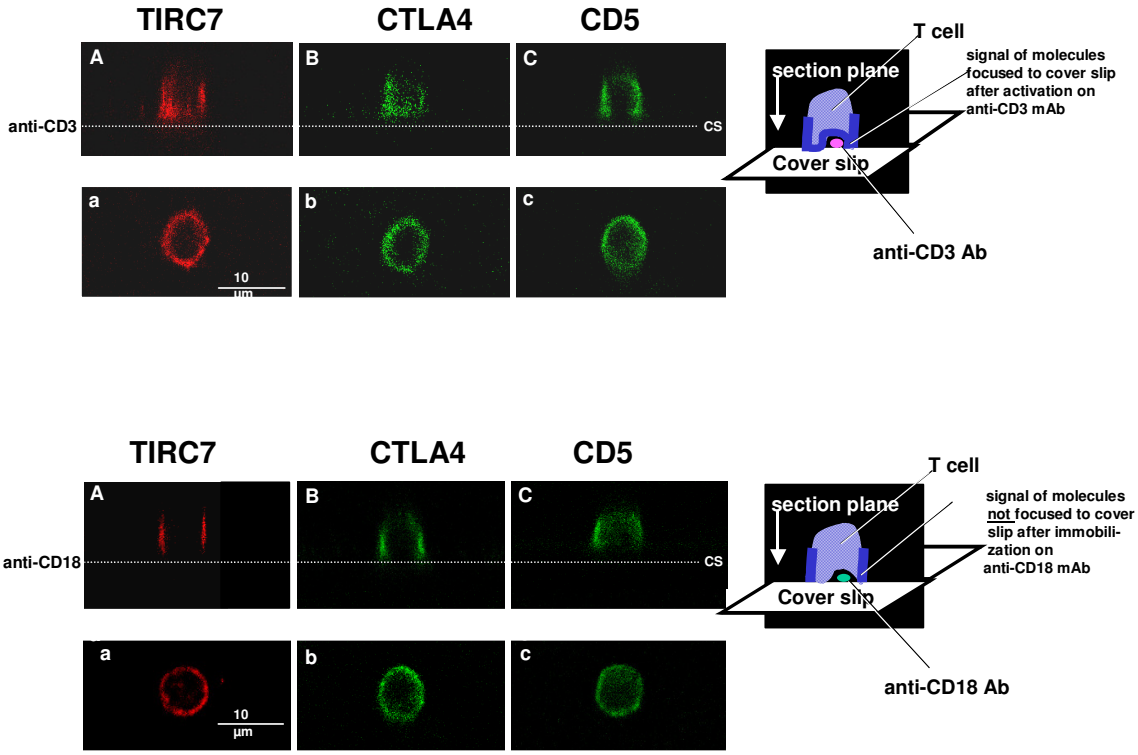
The analysis of TIRC7 co-localization with the TCR was performed using non-permeabilized human PBL immobilized on chambered cover slips, coated with either anti-CD3 mAb or anti-CD18 mAb, subsequently immune stained with anti-TIRC7, -CTLA-4, or - CD5 mAb. The results demonstrate that in cells stimulated with anti-CD3 antibody TIRC7 as well as CTLA-4 localized towards the antigen binding site (Fig. 2A (upper panel A and B). This localization was specific for the antigenic stimulus as TIRC7 and CTLA-4 did not show focal accumulation in lymphocytes immobilized using anti-CD18 mAb as shown in Fig. 2A (lower panel A and B). In contrast, a focal accumulation at the CD3 binding site was not observed for CD5 (Fig. 2A, upper and lower panel C). These results demonstrate that TIRC7, like CTLA-4, is specifically concentrated at the APC binding site upon antigen activation[41].

CTLA-4 has been previously demonstrated to be transported within clathrin-coated vesicles. The functional association between TIRC7 and CTLA-4 led us to hypothesize that the same mode of cellular transport would be observed for TIRC7. To examine this possibility, PBL were immobilized on chambered cover slips, permeabilized, co-stained with an anti-TIRC7 antibody and anti-clathrin heavy chain mAb, and the intracellular localization of TIRC7 compared with that of clathrin by confocal microscopy[41].

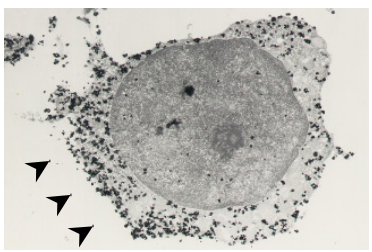
The results show that both proteins co-localize with clathrin coated vesicles and suggest that TIRC7 is transported towards and from the cell membrane via clathrin-coated vesicles, as was also described for several other costimulatory molecules. Moreover, the early appearance at the antigen adhesion site might also the result of reorientation of membrane-bound TIRC7 towards the immunologic synapse[17]. These findings may imply that TIRC7 plays a direct and critical role in the earliest events after engagement of T cells with antigens.

Figure 2. TIRC7 accumulates at the antigen binding site upon T cell activation.

(A) PBL were immobilized on anti-CD3 mAb or anti-CD18 mAb coated cover slips fixed in situ. TIRC7, CTLA-4, and CD5, were stained with the respective Cy3- and Cy2-coupled antibodies, and cellular localization was examined by confocal microscopy. A-C left upper panel show fluorescence imaging in vertical optical sections; the dotted line represents the antigen binding site (anti-CD3) on cover slip (CS). Upon activation TIRC7 accumulates in the antigen recognition site (CS) resulting in a cap-like image (upper panel A). The same pattern was observed for CTLA-4 (upper panel B). The corresponding horizontal sections of the cells, from which the vertical sections were taken, are shown below in a-c. The scheme at the right side of the upper panel depicts the frontal view of a T cell immobilized on an anti-CD3 mAb (pink dot, upper panel) or anti-CD18 mAb-coated (green dot, lower panel) coated cover slip (white layer). Along the vertical plane of the T cell (black plane) the fluorescing molecules which accumulate in a cap-like structure (dark blue line) towards the cover slip corresponding to cap-like structure of TIRC7 and CTLA4 (A and B, left upper panel). In contrast, a cap like distribution pattern was not displayed for CD5 (C, left upper and lower panel). All molecules immobilized on non-activating anti-CD18 mAb did not exhibit any focal accumulation to the CD3 biding site resulting in a uniform ring-like distribution pattern (lower panel A-C and a-c).



(B) PBL were immobilized and activated on anti-CD3 mAb coated cover slips for 24 h, tagged with TIRC7-coupled gold particles and analyzed by electron microscopy. The orientation of intracellular and surface TIRC7 towards the antigen binding site is indicated by arrowheads.



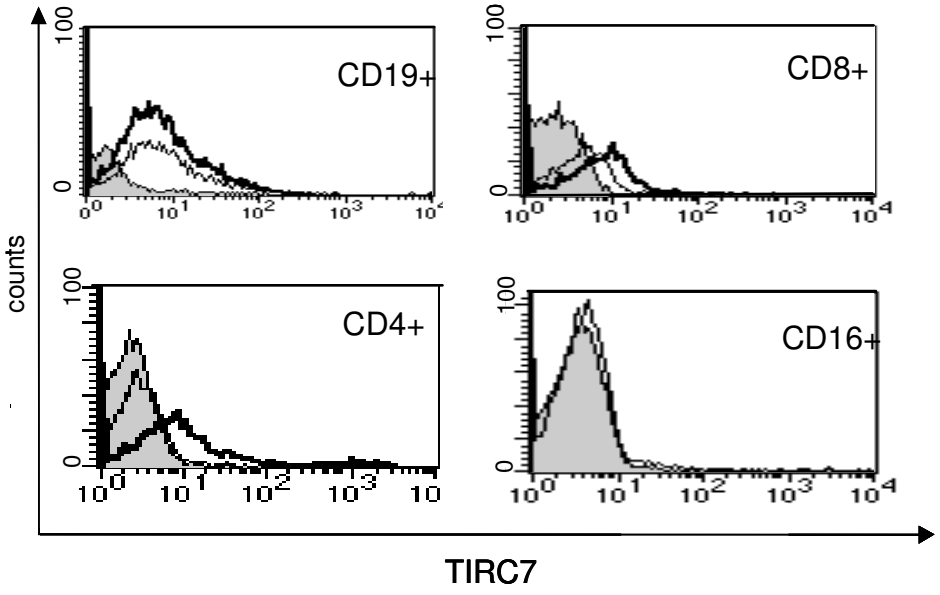
Another key observation is that a soluble form of TIRC7 protein inhibits T cell responses in human MLR cultures. Dose-dependent inhibition of T cell proliferation as well as cytokine production was observed in the cultures with recombinant, soluble TIRC7 proteins[40]. This observation strongly suggests the existence of TIRC7 ligand, most likely expressed on the antigen presenting cells, as is the case for all other T-cell co-stimulatory molecules.

3. 2. Expression kinetics of TIRC7 protein in human lymphocytes

TIRC7 protein is expressed predominantly in immune cells, primarily on activated T and B cells[42] activated with various stimuli, such as PHA or LPS+14. On PHA stimulation, slight upregulation of TIRC7 occurs in both CD4+ and CD8+ T cells (Fig 3). TIRC7 is expressed weakly in resting and activated CD19+ B cells, but not in CD16+ NK cells. Further studies revealed that TIRC7 bright cells are primarily enriched in the CD4+CD25+ T regulatory cell population, within the CD28+CD45RO+ memory T cell phenotype.

Figure 3. Expression of TIRC7 on activated human PBL

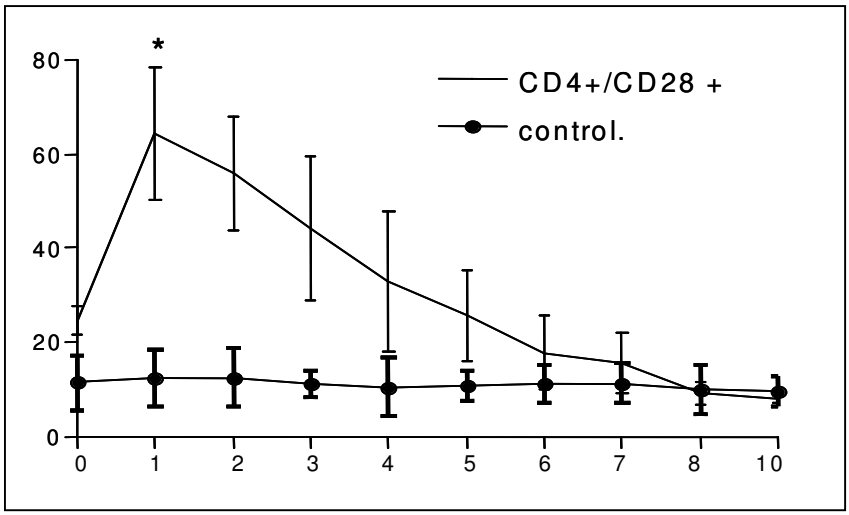
Flow cytometry analysis of human PBL after 18 h PHA or 48 h LPS and IL4 stimulation. Shaded grey line is the isotope control; light line resting cells; dark line activated cells.



The expression of TIRC7 is rapidly induced upon activation. When CD4+CD28+ T cells are stimulated with IL-2 or alloantigen, TIRC7 expression reached its peak level an hour after stimulation, and returned to baseline by 6 h[41] (Fig. 4).

Figure 4. Kinetics of TIRC7 expression on CD4+CD28+ T cells upon activation.

PBL were stimulated with 100 U/ml recombinant human IL-2. The kinetic of TIRC7 expression was determined by flow cytometry in human CD28+/CD4+ cells in comparison to control (x-axis=time (h), y-axis=MFI, maximal fluorescence intensity), (*P=0,005).



This rapid cell surface expression of TIRC7 occurs as a result of transportation of preformed TIRC7 protein stored in the endosomal compartment. Confocal microscopic investigation of human PBL demonstrated the subcellular localization of TIRC7 proteins in both cell surface and in cytoplasm. Flow cytometric analysis using non-permeabilized and permeabilized human PBL revealed the reciprocal changes in the subcellular localization of TIRC7 following mitogen stimulation, i.e., the maximum levels of cell surface expression at 1-2 h after stimulation, and concurrent decreased cytoplasmic expression[41].

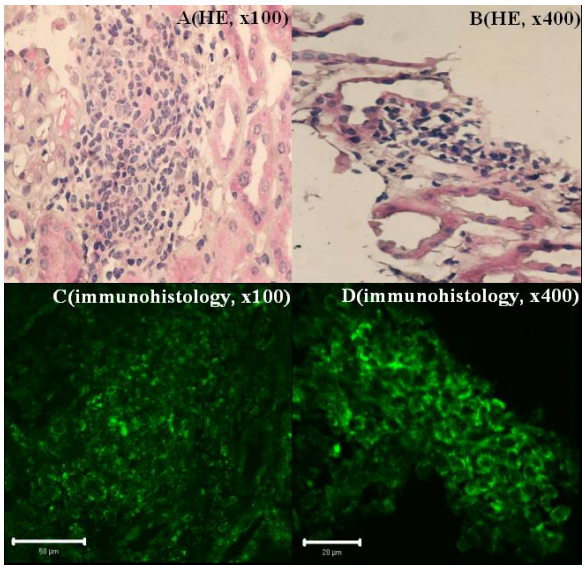
Northern analysis showed almost exclusive expression of the TIRC7 mRNA in immune tissues. High levels of mRNA expression was observed in the peripheral immune organs, including spleen, lymph nodes, PBL, and appendix, whereas lower levels of expression were detected in the bone marrow, fetal liver, and thymus. No signals were detected in other organs, including brain, heart, liver, kidney and lung[40].

3.3. TIRC7 expression in tissues from human diseases such as kidney transplant rejection, rheumatoid arthritis and multiple sclerosis

Pathological examination of biopsy specimen is most important to diagnose acute rejection, however, there are several difficulties as the transplanted organs are influenced not only by rejection, but also by infection, immunosuppressive agents, recurrence of original disease and other factors[34,35]. As the protein expression of TIRC7 reflects activation of the immune response and is not likely affected by immunosuppressive medication after the operation, measurement of the TIRC7 protein or mRNA expression might provide important information to diagnose acute rejection in early stage. Immune histological analysis of human kidney transplant biopsies utilizing a FITC labeled anti-TIRC7 mAb that were pathologically diagnosed as acute rejection shows strong TIRC7 expression (Fig. 5)[43]. TIRC7 mRNA expression was also induced within human heart allografts under rejection though it is down-regulated prior to rejection in peripheral blood mononuclear cells[44,45].

Figure 5. TIRC7+ mononuclear cells in human kidneys undergoing rejection.

Specimens obtained from patients (A, B) undergoing rejection show moderate to severe infiltration of lymphocytes reflecting acute rejection episode (HE staining). Examination of same specimen by immunofluorescent microscopy utilizing anti-TIRC7 mAb-FITC visualizes the expression of TIRC7 protein and its localised expression to the cellular infiltration areas (C, D).

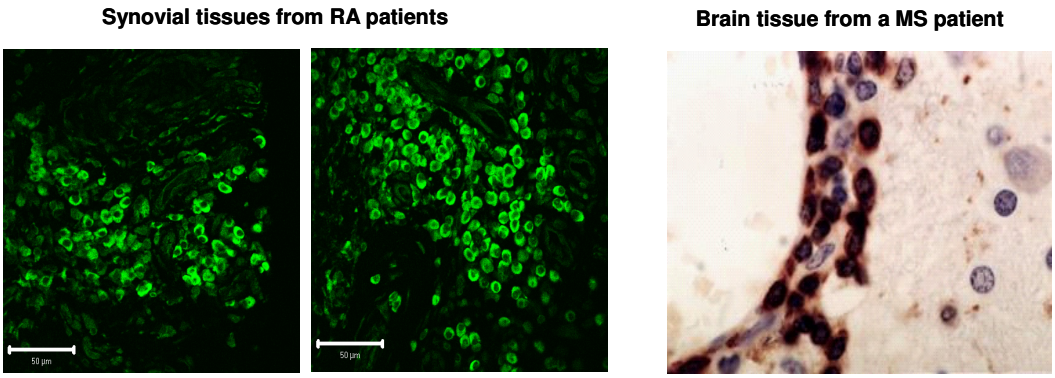


In an additional study, the kinetic mRNA gene expression profile of TIRC7 in the urine of kidney transplanted patients was investigated to gain more knowledge about the dynamics of this marker during acute rejection. mRNA obtained from urine samples (n=20) from renal-transplanted recipients was subjected to real time PCR analysis. Results were correlated with biopsy and creatinine data from each patient with acute rejection (n=10). During acute rejection patients displayed significant higher levels of TIRC7 mRNA in the urine compared to control patients with stable renal function (p=0.0017). Interestingly, induction of TIRC7 mRNA could be already observed six days prior a biopsy and indication of rejection by increased serum creatinine levels (p=0.005) (unpublished data). TIRC7 is also significantly induced in infiltrates obtained from joint or brain tissue of patients with either Rheumatoid Arthritis or with multiple sclerosis. Utilizing a directly labelled FITC anti-TIRC7 mAb in the immune histology resulted in:

- Marked accumulations of TIRC7+ cells detected in the synovial tissues of RA patients (Fig. 6, left)
- Significant perivascular accumulations of TIRC7+ cells in the neural tissues of MS patients (Fig. 6, right)

Figure 6. TIRC7+ lymphocyte infiltrations in RA synovial and MS brain tissues.

Immune histological staining using anti-TIRC7 mAb in tissue sections obtained from knee joint samples of patients with established RA (left and middle panel) for from brain tissue from patient suffering from MS (right panel).



Thus, changes of TIRC7 expression in biopsy specimens and peripheral blood might be useful markers not only for the diagnosis but also for the monitoring and prediction of allograft rejection after transplantation or autoimmune diseases.

3.4. Efficacy of polyclonal antibodies against human TIRC7 protein in vitro

Antibodies raised against TIRC7 effectively inhibit the proliferation of T cells induced by various stimuli, including PHA, ConA, anti-CD3 Ab, and alloantigens[40]. Rabbit polyclonal antibodies (Ab 73, 76 or 79) exhibited a markedly diminished T cell proliferation of PHA-stimulated human PBL[40].The suppressive effects were only partially overcome by exogenously provided IL-2. It was confirmed that the decreased proliferation signals observed in these cultures were not due to depletion of T cells. In addition, it was demonstrated that crosslinking of TIRC7 molecules by bivalent antibodies is required to induce inhibitory effects.

Anti-TIRC7 antibodies inhibited production of Th1 type cytokines in activated T cells *in vitro*[40]. Addition of rabbit polyclonal anti-TIRC7 antibodies to PHA-stimulated human PBL cultures strongly inhibited production of IFN-γ and IL-2, whereas Th2 cytokines (IL-4 and IL-10) as well as TNF-alpha were not affected. However, spleen cells isolated from TIRC7 null mice produced substantially higher levels of both Th1 and Th2 type cytokines (IFN- γ, IL-2, and IL-4)[46] suggesting a broader regulatory role of TIRC7 *in vivo*, which is not solely restricted to the Th1 pathway.

3.5. Efficacy of anti-TIRC7 polyclonal antibody in kidney transplantation in rats

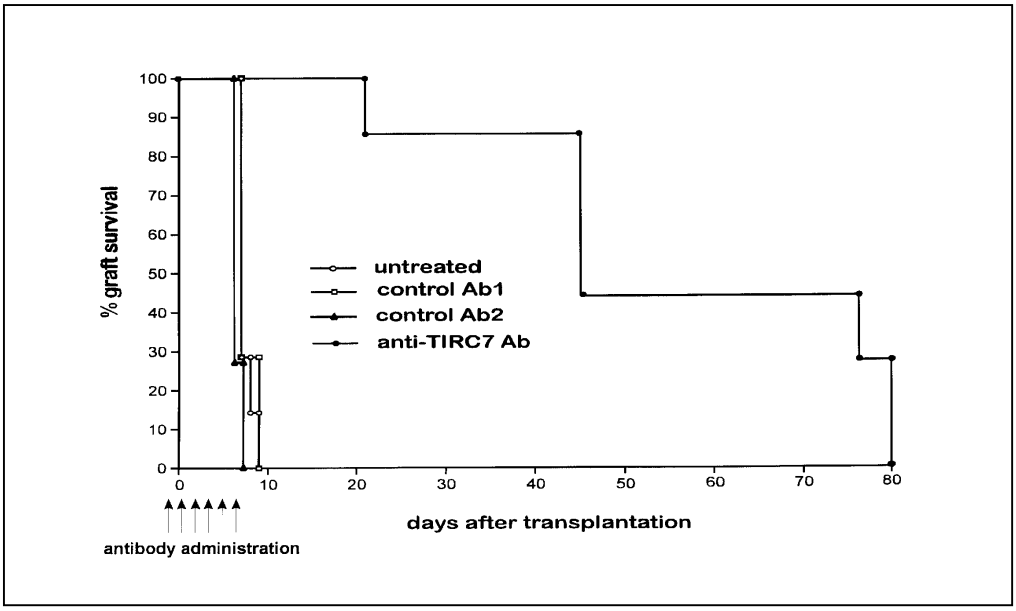
A rabbit polyclonal antibody targeting the extracellular domain of TIRC7 showed inhibition of rat T cell activation in an MLR[40]. This antibody was tested for its ability to prolong allograft survival in a model of Class I and II incompatible orthotopic kidney transplantation from Wistar Furth ($RT1^u$) to Lewis ($RT1^l$) rats. The antibody was given as an initial treatment 2 h before, directly after, and on days 1, 2, 4, 6 post-

transplantation (n=7). Anti-TIRC7 antibody significantly prolonged allograft survival (mean graft survival time=56 days compared to 9 days for controls; $p<0.01$)(Figure 7). Histological analysis of additional animals in each group sacrificed at day 7 demonstrated only mild lymphocytic infiltration in treated animals compared with severe infiltration and necrosis in control animals.

There was no depletion of CD3-, CD4-, and CD8- positive T cell subsets in peripheral blood at 24 h, day 2, or day 10 post-transplantation[40].

Figure 7. Antibody targeting of TIRC7 significantly prevents allograft rejection.

MHC class I and II incompatible orthotopic kidney transplantation from Wistar Furth ($RT1^u$) to Lewis ($RT1^l$) rats were performed and anti-TIRC7 antibody was given 2 h before, directly after, and on days 1, 2, 4, 6 post-transplantation (n=7). Anti-TIRC7 antibody significantly prolonged allograft survival (mean graft survival time=56 days) compared to 9 days for controls (Ab 1 and Ab2).



3.6. Immune responses in TIRC7 deficient mice

Results of *in vitro* and *in vivo* studies demonstrate that TIRC7 plays an important role in regulating T cell responses to a given antigenic stimulation. Results from two lines of experiments, analysis of the phenotype of TIRC7 null mice and *in vitro* studies with anti-

TIRC7 antibodies, collectively indicate that TIRC7 plays a critical role in the negative regulation of immune responses[40,41,42,43].

In order to understand the physiological functions of TIRC7 in the immune system, TIRC7 -/- mice were generated by a standard homologous gene targeting technology. In TIRC7 null mice, it was confirmed that the ratio of three genotypes of offspring falls within the expected Mendelian frequency[46].

TIRC7 null mice exhibited a significantly lower birth weight, followed by a retarded postnatal body growth, most likely due to the osteopetrotic changes in the bone structure. This abnormality can fully be explained by the lack of OC-116 expression, which is a splice variant of the TIRC7 gene[47,48,49]. TIRC7 and OC-116 share the last 14 exons and 13 introns.

OC-116 is a larger (96 kDa) protein with 8 transmembrane domains, with a predicted structure different from TIRC7. OC-116 is expressed exclusively in osteoclasts[48], and is suggested to play roles solely in bone metabolism.

The OC-116 null mice created by targeted deletion of exons 2-5 shares the phenotype of low body weight as well as osteopetrosis with the TIRC7 null mice[46,49]. However, they do not exhibit any abnormalities in the immune system.

No expression of OC-116 is detected in either activated or resting T cells[46,49]. Disruption of the TIRC7 gene resulted in overall hyperreactivities in both T- and B-cell responses to stimuli as summarized below (Table 1).

The observed phenotype of TIRC7 null mice serves as strong evidence to support the negative regulatory role of TIRC7 in a broad range of immune responses[46].

Table 1. Functional status of immune system in TIRC7 null mice

In vitro	▪ Increased T cell proliferation upon PHA or anti-CD3/CD28 stimulation ▪ Increased cytokine production (IFN-gamma, IL-2, and IL-4) by PHA stimulated splenocytes ▪ Increased B cell proliferation upon anti-CD40 or LPS+IL4 stimulation ▪ Increased production of IgM and IgG1 by stimulated B cells
In vivo	▪ Enhanced delayed type hypersensitivity (DTH) response ▪ Higher levels of serum Ig in all classes

3.7. Genomic organization of TIRC7

Genomic organization of the TIRC7 gene is composed of 15 exons and spans 7.9 kb[47]. The seven predicted transmembrane-spanning domains of the TIRC7 protein coincide well with exon-intron boundaries. TIRC7 and OC116, a recently described putative subunit of the vacuolar proton pump that was demonstrated to be expressed in an osteoclastoma tumor as well as in a human pancreatic adenocarcinoma cell line[48,49], are demonstrated to be alternative transcripts of the same gene. OC116 consists of 20 exons with the last 14 introns and exons being identical with those of TIRC7. The chromosomal locus for both transcripts was identified on chromosome 11q13.4-q13.5[47]. In alloactivated lymphocytes, only the expression of TIRC7, but not OC116, was detected, indicating that OC116 is not involved in lymphocyte proliferation.

3.8. Effects of the monoclonal antibody against TIRC7 in vitro

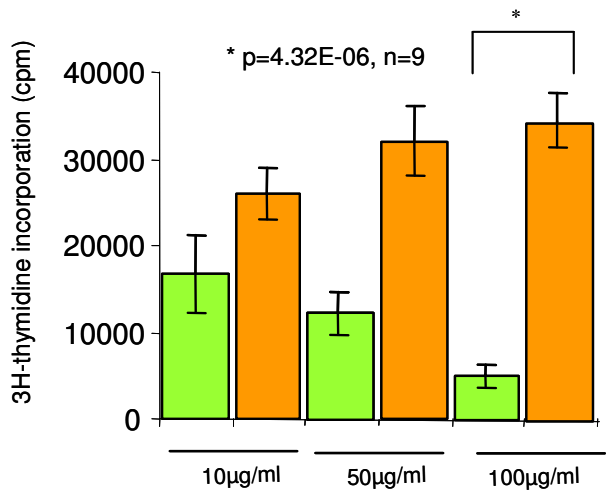
We have developed murine mAbs against human TIRC7 by immunizing mice with various synthetic peptides representing different domains of TIRC7 molecule (Fig. 1). An antibody against human TIRC7 molecules was selected that cross-reacts with murine TIRC7 allowing *in vivo* proof of concept studies with animal disease models.

For the clinical use a chimeric versions of the antibody was developed using its variable regions and combined with human Fc-IgG1. Anti-TIRC7 mAb was shown to bind to TIRC7 expressed on the surface of activated human T cells. Upon binding, the antibody acts as agonists, delivering inhibitory signals in activated T cells, which results in suppression of the immune response. The negative immune regulation mediated by the TIRC7 pathway appears to affect both T and B cell immune responses.

Anti-proliferative effects of anti-TIRC7 monoclonal antibodies were confirmed in various stimulation assays, including PHA, alloactivation, OKT3/CD28 and recall antigen assays. In the study presented in Figure 8, human PBL were stimulated with mitogen (PHA) for 48 h in the presence of anti-TIRC7 mAb (10, 50 and 100µg/ml) or a control mAb for 6 days. Anti-TIRC7 mAb treatment induced decreases in the of lymphocytes proliferation in a dose dependent manner.

Figure 8. Decreased proliferation in anti-TIRC7 mAb treated PBL

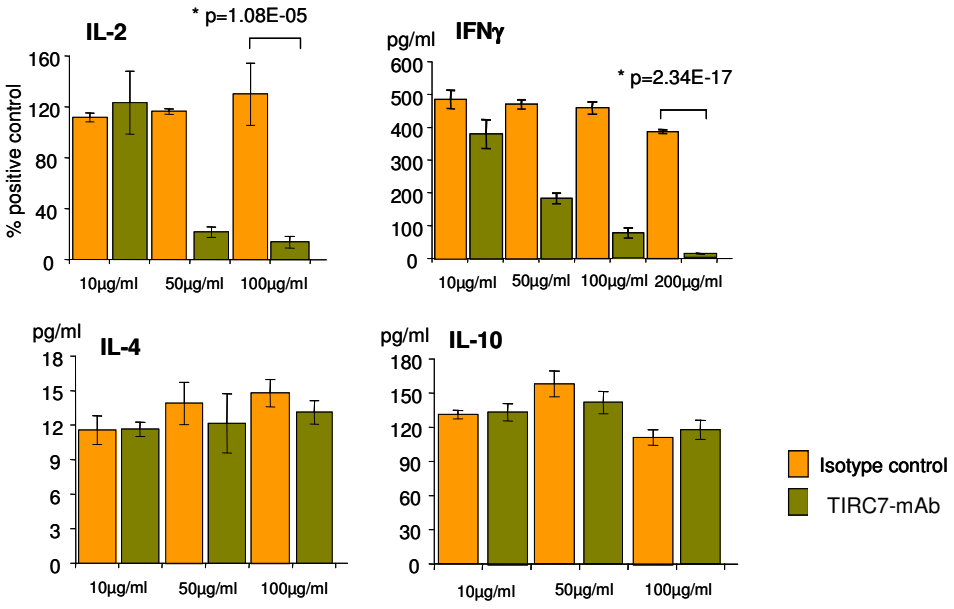
PBL were stimulated with PHA and incubated for 48h in presence of anti-TIRC7 mAb (green bar) or IgG-control mAb (orange bar). After 48h, 0,5 µCi ^{3}H-Thymidine per well were added and the cells were reincubated for additional 18h. Cells were harvested and scintillation counting was performed using a beta counter. Anti-TIRC7 mAb treated PBL show a significant inhibition of proliferation in a dose dependent manner.



In addition, inhibition of Th1 cytokine production was also confirmed using anti-TIRC7 mAb. In PHA-stimulated human PBL, anti-TIRC7 mAb inhibited IFN-gamma and IL-2 production in a dose-dependent manner (Fig. 9). In contrast, no suppression of IL-4, IL-10, or TNF-alpha was induced by anti-TIRC7 mAb.

Figure 9. Inhibition of cytokine expression in human PBL by antibodies against TIRC7

Anti-TIRC7 mAb inhibits significantly Th1 (IL-2; IFN-γ) cytokine expression of mitogen stimulated PBL after co-incubation of 48 h whereas Th2 associated cytokine expression (IL-10 and IL-4) remained unchanged. Detection of cytokines in the supernatants of activated PBMC was achieved using ELISA. Each bar represents six independent experiments.



Treatment of T cells with anti-TIRC7 antibodies also inhibited the expression of various activation markers, including CD25, CD69, and HLA-DR. On the contrary, these antibodies induced the expression of negative stimulatory molecule, CTLA4, in activated T cells. Collective evidence indicates that TIRC7 exerts its inhibitory action at least in part through upregulation of the CTLA4 molecule[41, 50]. In line with these observations, CTLA4 expression is markedly suppressed in activated T cells isolated from TIRC7 null mice[46].

4. EFFECTS OF MONOCLONAL ANTIBODY AGAINST TIRC7 IN VITRO AND IN VIVO

4. 1. In vivo pharmacology of a monoclonal antibody against TIRC7 protein

Unique immunosuppressive functions demonstrated by antibodies against TIRC7 make TIRC7 antibody therapy an option for the treatment of various immunological conditions, in particular autoimmune diseases and rejection of organ transplantation. *In vivo* efficacy of anti-TIRC7 mAb was evaluated with various murine and rat models for human diseases. Mouse models studied to date include acute organ rejection models and the collagen-induced arthritis (CIA) model for the treatment of RA. The chimeric anti-TIRC7 mAb was used in combination with FK506 in a rat transplantation model.

To identify the effects of anti-TIRC7 mAb as monotherapy in CIA and EAE models, the anti-mouse TIRC7 mAb was used since it avoids potential immune reactions of mice to the human Ig part in the chimeric mAb. It was confirmed that anti-TIRC7 mAb cross-reacts with murine TIRC7 and induces biological effects, including inhibition of T-cell proliferation and cytokine production[42,43,50]. Experiments described below were, therefore, designed with rather high doses of anti-TIRC7 mAb in order to prove the concept by demonstrating the *in vivo* efficacy.

4.1.a. Efficacy of anti-TIRC7 mAb therapy in allotransplantation

Management of alloreactive immune responses mediated by T cells is critical to ensure the successful graft survival in patients undergoing organ transplantation. Chronic treatment with calcineurin inhibitor immunosuppressants, such as Cyclosporin A (CsA) and Tacrolimus (FK-506), has significantly contributed in this regard; however, such therapies are associated with increased risks for infection and malignancies due to their prolonged, general immunosuppressive effects.

The unique effects of anti-TIRC7 mAb in inhibiting T cell activation on alloantigen stimulation provide a strong rationale to investigate its effects in preventing organ

rejection. It has been demonstrated that treatment with a rabbit polyclonal antibody reactive to human TIRC7 (which cross-reacts with rat TIRC7) significantly prolonged the graft survival in a rat kidney transplantation model[40]. The results of a study with a mouse cardiac transplantation model[50] as well as those of rat kidney transplantation model[40, 43] are presented here.

4.1. b. Effect of anti-TIRC7 mAb treatment in mouse cardiac transplantation

A model with the vascularized heterotopic cardiac transplantation performed in a fully-mismatched combination of C57BL/10 (H2b) donor and CBA (H2k) recipient was employed. In brief, donor hearts were transplanted by anastomosing the ascending aorta and pulmonary artery of the donor heart with the abdominal aorta and inferior vena cava of recipients using microsurgical technique. Graft survival was monitored by palpatation and the day of rejection was defined as the day the heart beat ceased. Without any treatment, transplanted hearts were rejected in average by 7.3 days in this combination[50]. Effects of anti-TIRC7 mAb treatment was investigated by intravenous (i.v.) anti-TIRC7 mAb administration 2 h prior to and immediately after surgery, followed by once daily administration on day 1, 2, 3, 4 and 7. Peri-operative anti-TIRC7 mAb treatment prolonged cardiac survival in all recipients with a significantly longer mean graft survival of 52.7 days ($p < 0.05$ vs. untreated controls), including two grafts that survived over 140 days[50].

Histological examination of the untreated grafts on day 7 revealed multifocal massive necrosis in the heart muscles, accompanied by inflammatory cell infiltration, perivasculitis and fibrosis. In contrast, the grafts of the anti-TIRC7 mAb treated group demonstrated only mild infiltration without destruction of the anatomical structure of the heart [50].

In immunohistochemistry analyses, a significant reduction of CD3+ T cells was evident in the treated heart tissues. Interestingly, a significant number of CTLA4+ cells were detected in the treated graft despite the fewer number of CD3+ T cells. Of note, CTLA4+ cells were not detected in the control grafts on day 7[50].

In order to understand the mechanisms underlying the prolonged graft survival, the functional status of T cells in anti-TIRC7 mAb treated animals was investigated using spleen cells of the recipient animals obtained on day 7. First, the reactivity to allogeneic stimulation was investigated by co-culturing with mitomycin (MMC)-treated spleen cells derived from C57BL/10 (donor) or Balb/c (a third party) mice. A statistically significant decrease in proliferation, measured by BrdU incorporation, was observed in the splenocytes derived from anti-TIRC7 mAb treated animals in response to donor cells as compared to that from untreated animals ($p < 0.007$). In contrast, no significant differences were observed in responses to third party cells. These results clearly indicated that anti-TIRC7 mAb treatment induced a donor-specific unresponsiveness in recipients' T cells *in vivo*[50].

Second, the ability of the splenocytes to produce cytokines on donor-specific antigen stimulation was investigated. The supernatants of co-cultures with MMC treated C57BL/10 splenocytes were harvested at 48 h and subjected to ELISA for cytokine measurements. Significant reductions were observed as compared with control animals in production of the acute phase pro-inflammatory cytokines, TNF-alpha ($p = 0.002$) and IL-6 ($p = 0.0002$), as well as a Th1 cytokine, IFN-γ ($p = 0.007$), whereas production of Th2 cytokines, IL-4 and IL-10 were not affected[50].

In conclusion, these results demonstrated the therapeutic benefits of anti-TIRC7 mAb treatment in suppressing organ rejection. Cases of long-term graft survival suggested that the donor-specific unresponsiveness induced by peri-operative anti-TIRC7 mAb treatment was maintained long-term without additional treatments. Thus, the selective

immune suppression uniquely induced by anti-TIRC7 mAb treatment may offer significant clinical advantages over current general immunosuppressants.

4.1.c. Synergistic effect of anti-TIRC7 mAb with FK-506 in rat kidney transplantation

In an earlier study, it was demonstrated that treatment with rabbit polyclonal antibodies reactive to human TIRC7 suppressed rejection of kidney allografts in a rat model system[40,41,51,52]. Using chimeric anti-TIRC7 mAb, synergistic effects between TIRC7 antibody treatment and a calcineurin inhibitor, Tacrolimus (FK-506, Prograf®) were demonstrated with a similar rat model of orthotopic kidney transplantation[43].

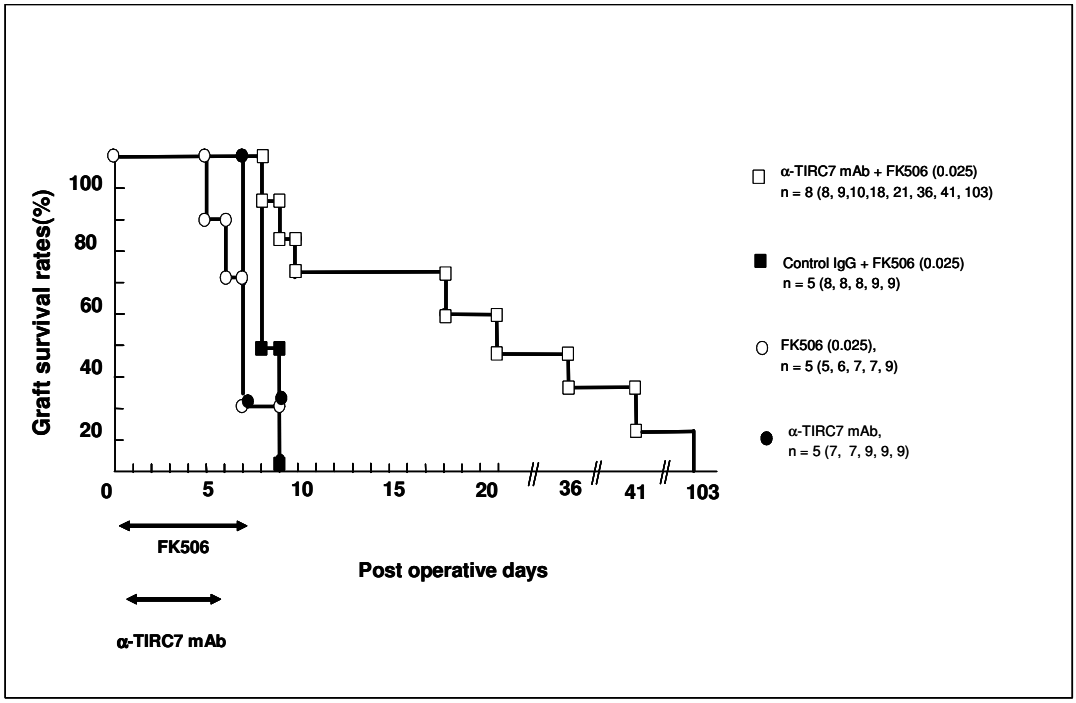
In brief, kidney allograft derived from D Agouti donor was grafted into bilaterally nephrectomized Lewis rat recipients by end-to-end anastomosis of left renal vessels and left urethra. Animals were treated either chimeric anti-TIRC7 mAb alone (2 mg/day i.v., starting from 2 h prior to operation, immediately after operation, and on days 1, 3, and 5), or Tacrolimus alone (0.025 mg/kg/day, i. m. for 14 days after operation), or with combination of chimeric anti-TIRC7 mAb and Tacrolimus, or with combination of Infliximab (Remicade®: chimeric IgG1 antibody reactive to human TNF-alpha, 2 mg/day as a control) or Tacrolimus alone(Fig. 10). The dose and protocol of each treatment was pre-determined such that the therapies would not have any effect in preventing organ rejection as a monotherapy.

Prolonged graft survival was observed in the group of rats treated with the combination of chimeric anti-TIRC7 mAb and Tacrolimus, while each monotherapy and combination of Infliximab and Tacrolimus were not effective. These results indicate the synergy of the combination of two treatments, which alone are not effective. Further, increased TIRC7 expression was detected in human kidney grafts undergoing rejection, in spite of immune suppressive therapies, including calcineurin inhibitors. Taken together, these findings suggest the possibility that the dose of Tacrolimus could be reduced by

combining with anti-TIRC7 mAb, thereby reducing the risks of adverse effects, such as nephrotoxicity.

Figure 10. Synergy between chimeric anti-TIRC7 mAb and Tacrolimus (FK 506) in suppressing kidney allograft rejection in rat model.

Application of FK506 (day 0 to 7) at a dose of 0.025 mg/kg showed no effect on graft survival (n=5). The monotherapy with the monoclonal antibody against human TIRC7 at a dose of 10 mg/kg for 5 times (day 0 x2, day 1, 3, and 5) was also not effective to prolong graft survival (n=5). The combination of these doses, however, prolonged the median graft survival up to 19.5 days (n=8). The combination of control mAb (Remicade®) and FK506 (0.025mg/kg) did not prevent graft rejection (p = 0.023 between anti-TIRC7 mAb and control IgG) (n=5).



5. ANIMAL MODELS FOR RHEUMATOID ARTHRITIS AND MULTIPLE SCLEROSIS

5.1. The collagen-induced arthritis (CIA) animal model in mice

Type II collagen-induced arthritis has been extensively used as a murine model for human RA. The disease is characterized by severe synovial inflammation that results in destruction of joint tissues and cartilage/bone erosions[53]. The immunopathogenesis of CIA involves both a B cell response against Type II collagen (CII) and a T cell response of

the Th1 type. Two different treatment protocols can be used with this model to assess the effect of a given therapy: 1) prevention model in which treatment is initiated prior to collagen immunization; and 2) therapeutic model in which treatment is initiated after the onset of clinical arthritis. Anti-TIRC7 mAb demonstrated efficacy in both prevention and therapeutic models as described in Utku et al[42]. In brief, male DBA/1 mice (8 week old) were injected intradermal with 100 μg bovine CII emulsified in complete Freund's adjuvant (cFA), at the base of the tail on day 0. On day 21, mice were boosted with 100 μg CII in incomplete Freund's adjuvant (iFA). Mice were then examined daily for the onset of CIA using two clinical parameters, paw swelling and erythema. The clinical score was graded[54] as 0 = no signs of erythema and swelling; 1 = erythema of fingers and digits or swelling of metacarpal or metatarsal joints; 2 = erythema and mild swelling of fingers and digits and/or metacarpal or metatarsal joints; 3 = erythema and severe swelling of fingers and digits and metacarpal or metatarsal joints; 4 = paws with deformity or ankylosis. Each limb was scored, giving a maximum score of 16 per mouse.

5.2. Therapeutic effects of anti-TIRC7 mAb therapy in CIA in mice

Anti-TIRC7 mAb or a control mAb was administered intraperitoneally (i.p.) at a dose of 500 μg/injection at day 24, three times/weekly for 60 days after the first immunization with collagen. Anti-TIRC7 mAb treatment exhibited significant therapeutic effects resulting in a delayed disease onset and progression with the maximum clinical scores maintained at around 2 as compared to those of control animals at 12. Results of histological (Fig. 12 left) as well as radiological (Fig. 12 right) examinations reflected the clinical therapeutic effects.

Figure 11. Effects of anti-TIRC7 mAb treatments on clinical scores in CIA.

DBA/1 mice were treated with 500 µg anti-TIRC7 or control mAb three times per week starting on day 24. Mice in the control group (*n*=7) developed typical clinical symptoms of arthritis which progressed rapidly to a mean arthritis score of 12 (orange curve). The group treated with anti-TIRC7 mAb (*n*=7) showed a significant reduction of the mean arthritis score and the maximal mean arthritis score the study was 2.5 (green curve).

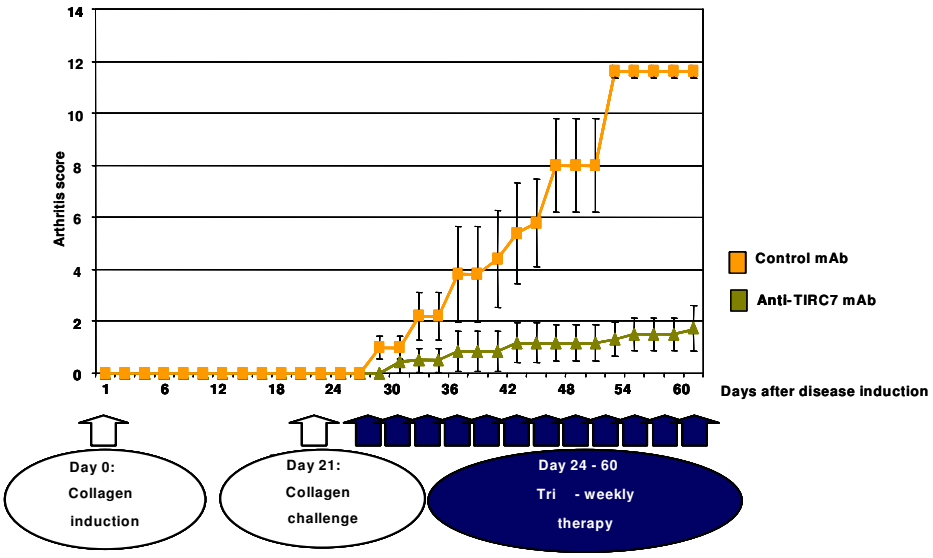
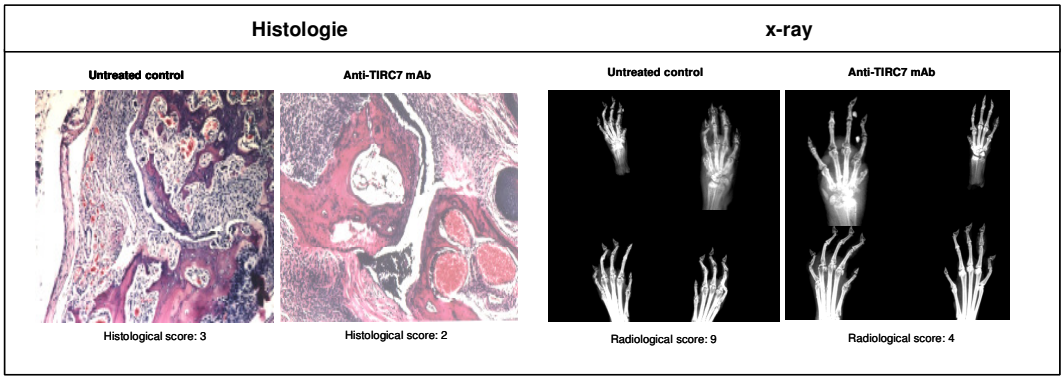


Figure 12. Effects of anti-TIRC7 mAb treatment on histology and x-ray in arthritis

Histology shows a marked disruption of joint structure with a loss of the articular surface, associated with pannus formation, and intense inflammation of the synovium in control mice (left). No histological abnormality recorded in the joint micro-architecture of anti-TIRC7 mAb mice (right). Radiographs of a control showed signs of arthritis with severe erosions disfiguration and osteolysis (left) whereas mouse treated with anti-TIRC7 mAb demonstrated neither lesions nor evidence of joint damage(right).



Similar effects of anti-TIRC7 mAb therapy were demonstrated when animals were treated at days after clinical score of 1, when clinical symptoms arose[42].

It has been demonstrated that anti-CII antibodies also contribute to the pathogenesis of CIA[54]. Therefore, serum levels of anti-CII antibodies were investigated by ELISA at the end of the studies. Interestingly, anti-TIRC7 mAb treatment resulted in decreases in anti-CII antibodies, in both IgG1 and IgG2a classes. The decrease in IgG1 was statistically highly significant ($p=0.00196$). This result, together with the results obtained in TIRC7 null mice, may imply that the TIRC7 pathway mediates inhibitory effects in both T and B cell responses *in vivo*.

5.3. Experimental autoimmune encephalitis (EAE) model in mice

Experimental autoimmune encephalomyelitis is a CD4+ Th1-mediated demyelinating disease of the central nervous system (CNS) that serves as a standard model for human MS. EAE is characterized by progressive ascending paralysis resulting from infiltration of antigen-specific and non-specific CD4+ and CD8+ T cells as well as macrophages[55]. EAE can be induced in a number of genetically susceptible animal species by immunization with myelin or the myelin components, including myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG)[56]. Similar to the CIA model, two different treatment protocols can be used with this model to assess the effect of a given therapy: 1) prevention model in which treatment is initiated after immunization but prior to disease manifestation; and 2) therapeutic model in which treatment is initiated after disease onset. The initial results with both preventive and therapeutic models are described here, since preventive effects may be clinically relevant considering the relapsing-remitting nature of MS. In brief, female SJL/J mice (8 week old) were immunized with 150 µg of PLP 139-151 peptides subcutaneous (s.c.) and 400 ng pertussis toxin i.p. on day -2, and with 400 ng pertussis toxin i.p. repeated on day 0. Animals were examined daily for signs of disease and graded with the clinical score as 1 = limp tail or limb weakness; 2 = limp tail and limb weakness; 3 = partial hind

limb paralysis; 4 = complete hind limb paralysis; 5 = moribund state. The maximum score per animal is 5. In this protocol, disease manifestation occurred at around day 12.

5. 4. Therapeutic effects of anti-TIRC7 mAb in EAE in mice

Efficacy of anti-TIRC7 mAb in treating established EAE was investigated by initiating the treatments after animals manifested the clinical score of 1. Animals were treated daily with anti-TIRC7 mAb for 7 days, and thrice weekly thereafter, for a total of 15 days. Untreated control group showed a typical relapsing-remitting EAE curve, while clinical scores of anti-TIRC7 mAb treated group remained around 1 or lower. The results indicated that anti-TIRC7 mAb treatment halted disease progression with some alleviating effects on the established EAE (unpublished data). Histological analyses of the brain and spinal cord revealed dramatic decreases in infiltration of mononuclear cells in these tissues, resulting in a significant decrease in the histological scores. Representative histologies demonstrate the marked accumulation of mononuclear cells in the periventricular space in the control animals in contrast to the limited cellular infiltration in the anti-TIRC7 mAb treated animals.

6. DISCUSSION

One of key objectives in the field of immunology is to develop new, specifically acting therapeutics without the adverse effects of today's chronic immune suppression therapeutics to treat organ transplantation rejection and autoimmunity.

The DDRT-PCR analysis we have performed aimed to identify differentially and early expressed proteins in human lymphocytes that might serve as targets for therapeutic intervention to specifically treat undesired immune activation. The secondary aim of our study was to analyse the expression profile in detail of these differentially

expressed targets in various immune activation settings to develop diagnostic markers to predict immune activation at early stages in immune mediated diseases.

The results obtained from this study demonstrates the isolation of the novel membrane protein, TIRC7, which represents a protein with a dual role, on one hand as a target and on the other hand as diagnostic marker for therapeutic and diagnostic interventions of immune mediated disorders [40,41,42,43,44,45,46,47,50,51,52].

TIRC7 expression in tissues

We have described in this study the isolation of a previously unknown protein, called T cell immune response cDNA7 (TIRC7)[40]. This molecule is specifically expressed in certain subsets of T and B cells of human lymphocytes.

Confocal microscopy studies demonstrated that the cell surface distribution of TIRC7 molecules is enriched towards antigen binding sites and that TIRC7 co-localizes with the T cell receptor/CD3, indicating the functional involvement of TIRC7 in immune activation events[41].

Moreover, TIRC7 is upregulated in infiltrating mononuclear cells in human kidney allografts that undergo rejection[43], in infiltrates of synovial tissues of Rheumatoid Arthritis patients[42] and in infiltrates of neural tissues of Multiple Sclerosis patients.

In a recent study, we observed that the TIRC7 protein is strongly expressed in kidney biopsy tissues from patients with acute transplant rejection in spite of strong immunosuppressive medication, i.e., TIRC7 expression is not down-regulated by CNIs in lymphocytes in the tissues undergoing organ rejection[43]. Immunohistological comparative analysis of TIRC7 and CD3 expression in kidney tissues indicates that TIRC7 expression is solely restricted to mononuclear cell infiltrates in kidney allografts. These findings confirm a report of Shulzhenko et al.[44], demonstrating increased mRNA expression of TIRC7 in rejected allograft tissue.

In an additional study, the kinetic mRNA gene expression profile of TIRC7 in the urine of kidney transplanted patients was investigated to gain more knowledge about the dynamics of this marker during acute rejection. Interestingly, induction of TIRC7 mRNA could be already observed six days prior to biopsy, which suggests TIRC7 might serve as a candidate marker with predictive properties to uncover inflammatory changes within the graft following kidney transplantation.

These results strongly suggest the pathogenic role of TIRC7+ cells in these diseases, indicating a potential therapeutic as well as diagnostic role of TIRC7 pathway.

TIRC7 antibody targeting results in inhibition of Th1 cytokine response

To explore the functional role of TIRC7 protein, antibodies against different peptide epitopes were generated and utilized in various *in vitro* and *in vivo* assays. Several rabbit and murine antibodies against human TIRC7 protein were obtained by immunizing with various synthetic peptides representing different domains of TIRC7 molecule[40,50].

The functional domains, initially identified assuming a hypothetical structure of the TIRC7 amino acid sequence, were confirmed by studies using antibodies. Only antibodies recognizing either the large extracellular- or C-terminal domain of TIRC7 exhibited significant inhibition of human, rat and mice lymphocyte proliferation, in mitogen as well as allo-antigen stimulation assays. The inhibition of the immune response was achieved at significant lower doses when the antibody was cross-linked to culture plates, indicating that cross-linking is required to activate TIRC7 signaling pathway.

Inhibition of Th1 cytokine production was confirmed in mitogen stimulated human PBL, which showed an inhibition of IFN-γ and IL-2 production in a dose-dependent manner. In contrast, no suppression of IL-4, IL-10, or TNF-α was induced by anti-TIRC7 antibodies[40,50]. The inhibition of proliferation was reversed through adding of recombinant IL-2. However, IFN-γ was only partially abolished, indicating that the anti-TIRC7 mAb is able to induce a stable Th1 cytokine inhibition.

Antibody targeting of TIRC7 results in prevention of solid organ transplantation

Since the introduction of calcineurin inhibitors, organ transplantation has been the most effective therapy for the end-stage patients of various acute and chronic organ failures. CNIs exert strong immunosuppression. However, continuous clinical administration is associated with toxicities on the kidney and the nervous system, and often with the development of malignancies[3,4]. As CNIs are currently the major immunosuppressive agents in clinical use, many studies have been carried out exploring reduction of CNI doses by combination with other immunomodulatory compounds[29,30,35].

In current clinical practice, certain antibodies are applied as induction treatment of acute rejection in addition to calcineurin inhibitors. A monoclonal antibody against CD3 (OKT3)[33] as well as the anti-lymphocyte antibody (ATG)[31,32] are available, however, they have severe side effects, such as cytokine release syndrome, serum sickness and lymphocytopenia.

There are several reports of antibody or protein therapy targeting costimulatory pathways, such as CTLA4:CD80/86 and CD154:CD40 that are required to initiate T cell activation[6,11]. Two monoclonal antibodies against the IL-2 receptor, basiliximab and daclizumab, were developed showing beneficial effects in clinical trials by reducing acute rejection as a consequence of induction therapy[36]. However, these mAbs are not sufficient to prolong allograft survival when used as monotherapy. Thus, the high unmet need to reduce the strong immunosuppressive effects of CNIs still remains.

TIRC7 protein exhibits significant homology between both rodents and humans[42,43,46,50]. Using antibodies that cross-react with mouse and human TIRC7, we demonstrated that TIRC7 targeting significantly inhibits Th1 cytokine expression and prolongs kidney graft survival in rat[40]. Similar to results obtained in rat transplantation, the peri-operative application of an anti-TIRC7 mAb diminished CD3 lymphocyte infiltration into the cardiac allograft[50]. Moreover, splenocytes from mice treated with the anti-TIRC7 mAb

exhibited prolongation of hyporesponsiveness to the donor allo-antigen or revealed decreased IFN-γ and TNF-α production but sparing inhibition of IL-4 and IL-10 production. These effects of TIRC7 ligation are similar to those described for other co-stimulatory molecule pathways, such as CTLA-4:CD80/86 and CD154:CD40. However, TIRC7 does not share sequence or structural homology with any known co-stimulatory proteins expressed on T or B cells.

Cell surface marker analysis on lymphocytes treated with anti-TIRC7 mAb *in vitro* showed alteration of the expression of activation markers, including the down-regulation of CD25 and up-regulation of CTLA-4. In addition, immunohistological analysis also revealed CTLA-4 up-regulation on graft infiltrating lymphocytes in anti-TIRC7 mAb treated grafts[50]. These results suggest that early and sustained upregulation of CTLA-4 induced by mAb against TIRC7 results in transduction of negative signals to allo-activated T cells inducing hyporesponsiveness to the allo-antigens.

Introduction of a new compound in the field of clinical transplantation might be especially promising if combined with a calcineurin inhibitor. It might be, therefore, desirable to demonstrate that the functional pathway of the new investigational drug is not affected by the treatment with CNIs after transplantation. To this end, we investigated a combination therapy of anti-TIRC7 mAb and a calcineurin inhibitor, FK506, in a rat kidney transplantation study[43]. This therapeutic combination prolonged the median graft survival up to 19.5 days ($p<0.023$ between anti-TIRC7 mAb + FK506 and control IgG + FK506) indicating the synergistic effect of both. These results indicate that the combination of both compounds might be a useful therapeutic approach in clinical settings to reduce the dose of CNIs after transplantation.

TIRC7 antibody targeting significantly prevents progression of arthritis in mice

Many autoimmune diseases, such as Rheumatoid Arthritis are associated primarily with an exaggerated Th1 response[57,58,59]. Since specific modulation of the T cell response

remains a so far unresolved goal in the field of immunotherapy those autoimmune diseases remain a major health problem despite of significant efforts to understand the underlying pathogenetic mechanisms. A lack of clarity with regard to both, the predisposing factors and the precise antigenic targets, have restricted the development of effective therapeutic approaches[59]. Recent data suggest that, in addition to the (auto) antibody production, B cells contribute significantly to the pathogenesis of many autoimmune diseases[60, 61]. These findings suggest the need for specifically affecting both T and B cells in achieving an appropriate treatment of autoimmune diseases.

Rheumatoid Arthritis, a common human autoimmune disease, is characterized by a chronic inflammatory reaction in the synovium of joints that is associated with cartilage degeneration and juxta-articular bone erosion[59]. The histopathologic features of synovitis involve a massive leukocyte infiltration consisting primarily of macrophages and CD4+ T lymphocytes but also of B cells[60,61]. The conventional therapy and the more recent selective anti-inflammatory therapy targeting TNF-alpha and IL-1 interact with late effector mechanisms of the disease resulting in high efficiency but limited long-term success. Moreover, long-term neutralization of effector cytokines compromises anti-infectious response[58].

In T cells, TIRC7 is predominantly upregulated on CD4 and CD8 positive memory T cells. Compared with intracellular levels of TIRC7 the surface expression of TIRC7 is relatively low in T cells. This expression profile of TIRC7 with predominance in memory T cells is also reflected by the strong inhibition of proliferation obtained with anti-TIRC7 mAb in T cells stimulated with recall antigen. However TIRC7 is also present in resting and activated B cells.

Anti-TIRC7 mAb treatment exhibited significant therapeutic effects resulting in a prevention of disease progression in the CIA model. Results of histological as well as radiological examinations also demonstrated significant therapeutic effects of anti-TIRC7 mAb in treating established CIA[42].

In addition, antibody targeting of TIRC7 also affects B cell activation as assessed by the significant reduction of the levels of both IgG1 and IgG2a anti-collagen antibody in the CIA model. These results are in line with previous observations made in B lymphocytes obtained from TIRC7 deficient mice[46] that showed elevated cytokine secretion and increased immune activation, suggesting lack of inhibitory influence on lymphocyte activation. The results of a down-regulation of both classes of antibody responses rather than a specific effect on only IgG2a responses suggest an independent significant effect of anti-TIRC7 antibody treatment on B cell responses. Thus, TIRC7 targeting might be of particular interest as a novel therapeutic approach in the treatment of RA. The effect of anti-TIRC7 mAb on B cell activation might lead to reduction of pathologic antibody responses, such as immunogenicity to biologic compounds, which suggests that its efficacy may be broadly applicable for this class of disorders.

If translated into clinical use, the unique mechanism of action and expression dynamics of TIRC7 might offer a promising novel target to treat rejection as well as RA and other autoimmune disorders.

Antibody targeting of TIRC7 prevents progression of EAE in mice

The majority of the perivascular mononuclear cells in brain tissue from MS patients were found to be TIRC7-positive T cells. TIRC7 expression was also increased in other cell types such as in B cells, notably in the activated microglia in inflammatory MS lesions whereas resting microglia in normal brain were TIRC7 negative. This indicates that upregulation of TIRC7 protein requires antigen stimulation.

Given the likely role of mononuclear cells in MS and that activated T cells in MS lesions express TIRC7, the functional effects of TIRC7-modulating therapy in an EAE animal model of MS was investigated. Administration of anti-TIRC7 mAb reduced the clinical and pathologic severity of EAE in mice by inhibition of mononuclear cell infiltration into

the CNS and by suppressing selective cytokines, such as IFN-γ and IL-6 (unpublished data).

The effectiveness of currently used immunomodulatory therapies, such as IFN-β in the majority of MS patients is not sustainable[62]. Therapies designed to circumvent auto-activation of CD4+ cells and the resulting release of cytokines involved in tissue damage, such as anti-CD3 cell therapies were ineffective. Therapies directed against the neuroprotective or detrimental cytokines themselves, such as tumour necrosis factor α or β were either toxic or exacerbated the disease[63, 64]. However, the approach of using mAbs against various elements of the MS process, such as CD52 or alpha4 integrin[65] has shown to be a major therapeutic improvement.

Reactivation of autoimmune diseases, such as rheumatoid arthritis and MS can occur through the development of new auto-antigens that elicit activation and, in the case of MS, CNS infiltration of Th1 cells that recognize the progressing auto-antigen repertoire[66,67]. Thus, these findings raise the possibility that anti-TIRC7 mAb therapeutic intervention may suppress inflammation at the onset and in exacerbations of relapsing-remitting MS.

TIRC7 delivers negative signals to lymphocytes

The deletion of TIRC7 resulted in both T and B cell hyperresponsiveness suggesting that TIRC7 is normally involved in the negative regulation of these cell types[46]. The latter result that B cell hyperresponsiveness is observed in the absence of TIRC7 expression is consistent with recent preliminary data that TIRC7 is also expressed by B cells. This suggests that TIRC7 may also provide negative regulatory functions for B cells through signaling pathways that are yet to be defined. The data obtained from TIRC7 deficient mice also help to explain previous studies obtained with polyclonal and monoclonal antibodies directed against the TIRC7 molecule.

Targeting of the TIRC7 molecule with polyclonal antibodies was previously shown to

inhibit both proliferation and Th1 cytokine secretion. This effect required cross-linking of TIRC7 as Fab fragments did not mediate these inhibitory effects (unpublished observation). These data could not distinguish whether TIRC7 is a stimulatory or inhibitory molecule as the antibodies used could have either reflected a ligand-triggered, negative signal provided by TIRC7 or blocked a positive signal induced by the physiological TIRC7 ligand. The enhanced T cell proliferation and Th1 cytokine secretion observed in TIRC7 knock out mice following different T cell stimuli suggest that TIRC7 normally negatively regulates the activation of T cells.

However, in contrast, to our previous report that TIRC7 specifically regulates Th1 cytokine secretion, studies with the TIRC7-deficient mice show that, both Th1 and Th2 cytokine responses are affected. This may be due either to differences between rodent and human T cells or reflect a threshold effect in that Th1 responses are more sensitive to Th2 responses. It is also possible that different agonistic signals delivered by TIRC7 as modelled by antibody-mediated ligation may differentially regulate Th1 vs. Th2 cytokine secretion.

TIRC7$^{-/-}$ mice exhibited a diminished up-regulation of CTLA-4[46]. Both, membrane and intracellular levels of CTLA-4 were reduced during T cell activation in TIRC7-deficient mice. Although the increased immune response of splenocytes obtained from TIRC7$^{-/-}$ mice exhibit some similarities to CTLA-4 deficient mice, the phenotypes are not completely comparable. In contrast to CTLA-4$^{-/-}$ mice, no significant enhancement of spontaneous T cell proliferation *in vitro* and much less evidence for an enhanced autoimmunity *in vivo* was observed in TIRC7 deficient cells. The fact that CTLA-4 was not completely abolished in TIRC7$^{-/-}$ mice may explain the less severe phenotype in TIRC7-deficient mice. However, TIRC7$^{-/-}$ mice share with CTLA-4 deficient mice an enhanced spleen/body weight ratio, which might be due to marked increased in granulocytopoiesis observed in the case of TIRC7-deficiency. Although the lymphocyte hyperactivity is less severe in TIRC7$^{-/-}$ mice in comparison to CTLA-4$^{-/-}$ mice, this does

not rule out the possibility that TIRC7 controls T cell activation, at least in part, via CTLA-4 up-regulation. It is also likely that other regulatory pathways are triggered by TIRC7 besides CTLA-4.

These results provide strong support for the assumption that TIRC7 is involved in the regulation of receptor molecules that are essential for a balanced T and B lymphocyte mediated immune response. Our studies, using antibody-mediated ligation as a model, suggest that antibody ligation of TIRC7 mediates the induction of lymphocyte hypo-responsiveness, possibly leading to an anergic state of lymphocytes. Consistent with this, TIRC7-deficiency leads to lymphocyte hyperresponsiveness.

The results obtained from TIRC7 deficient cells might not only contribute to a basic understanding of lymphocyte stimulation but might also have substantial clinical implications by suggesting that targeting of TIRC7 to specifically induced hypo-responsiveness in lymphocytes may be a therapeutic modality for immune activation related diseases.

TIRC7 null mice exhibited a significantly lower birth weight, followed by a retarded postnatal body growth, most likely due to the osteopetrotic changes in the bone structure. This abnormality can fully be explained by the lack of OC-116 expression, which is a splice variant of the TIRC7 gene[47]. TIRC7 and OC-116 share the last 14 exons and 13 introns. OC-116 is a larger (96 kDa) protein with 8 transmembrane domains, a predicted protein structure different from TIRC7. OC-116 is expressed exclusively in osteoclasts and is suggested to play roles solely in bone metabolism. The OC-116 null mice created by targeted deletion of exons 2-5 shares the phenotype of low body weight as well as osteopetrosis with the TIRC7 null mice[46, 49]. However, the OC-11 knock out mice do not exhibit any abnormalities in the immune system [49]. No expression of OC-116 protein is detected in either activated or resting T cells, however, several splice variants are described to be expressed on the mRNA level[68]. However, biochemical studies to analyze different splice variants at the protein level using antibodies against

various domains of TIRC7 that share sequential homology with OC-116 or other splice variant revealed solely TIRC7 protein, indicating that TIRC7 is the only translated protein in lymphocytes.

TIRC7 mediates negative signals via CTLA4 signaling pathway

Although detailed mechanisms of T-cell hyporesponsiveness following TIRC7 mAb treatment are yet not fully understood, the effects of TIRC7 ligation are quite similar to those described by targeting other co-stimulatory molecule pathways, which suggests that TIRC7 might mediate negative co-stimulatory signals. Consistent with this, lymphocytes from TIRC7 deficient mice exhibit signs of immunological hyperactivity, such as enhanced proliferation as well as cytokine and antibody secretion following *in vitro* stimulation. These data thus support a negative signaling role for TIRC7 in the context of immune cell activation.

In this regard, anti-TIRC7 mAb treated allograft recipients exhibited an intragraft up-regulation of CTLA-4[50]. In order to address the possibility that CTLA-4 was up-regulated on T cells after TCR stimulation in the presence of anti-TIRC7 mAb, we analyzed the cell surface markers expressed on lymphocytes activated in the presence or absence of anti-TIRC7 mAb *in vitro*. Anti-TIRC7 mAb treatment altered the expression of several surface markers, including down-regulation of CD25 (IL2R) [40, 50] and up-regulation of CTLA-4[41, 50]. This suggests that sustained up-regulation of CTLA-4 as induced by anti-TIRC7 mAb treatment results in transduction of negative signals to allo-activated T cells inducing hyporesponsiveness to the stimulatory allo-antigens.

Moreover, the reversal of effects of anti-TIRC7 antibody induced-T cell hypo-responsiveness and the inhibition of Th1 specific cytokine production by antibody blockade of CTLA-4[41] strongly suggests that the inhibitory effect of TIRC7 on T cells is executed mainly via CTLA-4. The up-regulation of CTLA-4 mRNA in human T cells upon TIRC7 antibody targeting and the early and sustained up-regulation of CTLA-4 expression

on the cell surface within only few hours indicate a close regulatory link between both molecules and strongly suggest that TIRC7 functions as an upstream molecule in a negative signaling cascade involving CTLA-4.

Interestingly, *in vivo*, CTLA-4 up-regulation on lymphocytes was only observed in graft infiltrating lymphocytes, and not on peripheral blood lymphocytes or splenocytes at day 7 post-transplantation suggesting that the effect of TIRC7 ligation is directly dependent upon the presence of the allostimulus[50]. Moreover, antibody targeting of TIRC7 has the potential to open the door to tolerance induction as well as being a promising partner with CNIs in a combination therapy that permits reduction of their dose and consequently their side effects in transplantation.

Identification of the ligand to TIRC7

The cell surface expression profile and antibody blocking experiments suggested the presence of a ligand interacting with TIRC7 at the cell surface.

Using the large extracellular domain of TIRC7 in a yeast two hybrid system, we identified the human alpha 2 domain of HLA-DR as domain binding to TIRC7. This binding was confirmed via co-precipitation of the HLA-DR alpha 2 from lysates of alloantigen activated human peripheral mononuclear cells using an anti-TIRC7 mAb (unpublished data). Further binding studies via FACS analysis showed a dose dependent binding of soluble HLA-DR alpha 2 fusion protein (sHLA-DR a2) to TIRC7 protein expressed on the membranes of transfected COS7 cells. The peptide motif of TIRC7, which interacts with HLA-DR alpha 2 domain was identified and shown to bind solely to HLA-DR positive cell lines. Binding was abandoned when mutations were introduced into the relevant TIRC7 peptide motif.

Moreover, in functional *in vitro* assays, incubation of activated human T cells with sHLA-DR a2 resulted in a significant inhibition of proliferation and IFN-γ production, whereas Th2 cytokines remained unchanged. The binding of human sHLA-DR a2 to mouse TIRC7

protein was confirmed using mice splenocytes whereas no binding was observed using splenocytes from TIRC7 deficient mice.

Soluble HLA-DR a2 exhibits an anti-proliferative effect on mice splenocytes via binding to TIRC7 protein as no inhibition of proliferation was observed in TIRC7 deficient mice splenocytes. The analysis of the ability of sHLA-DR a2 to suppress the *in vivo* immune response showed strong inhibition of IFN-γ expression in a LPS-induced mice model, which strongly supports the functional relevance of the binding between TIRC7 and sHLA-DR a2. These results suggest that the interaction of TIRC7 and HLA-DR alpha 2 might provide a novel therapeutic approach for the treatment of immune mediated diseases.

In summary, a novel biologic pathway involving TIRC7 and HLA DR has been discovered. By use of biological agents (antibodies and soluble ligands) it has been shown that the modulation of this pathway may provide clinical benefits in the treatment of autoimmune diseases and transplanted organ rejection.

7. SUMMARY

The membrane protein, TIRC7 (T cell immune response cDNA7) was identified as a molecule transiently expressed in T cells following activation by mitogens or allo-antigens via DDRT-PCR analysis.

Results of several *in vitro* and *in vivo* studies, including those with TIRC7 null mice, indicate that TIRC7 is a key negative T cell regulatory molecule upstream of other inhibitory pathways. Signals downstream to TIRC7 induce suppression in both T and B cell immune responses. TIRC7 molecule has been shown to serve as potential immunoregulatory target for the development of novel therapies for various immunological disorders.

The scientific findings around TIRC7 generated to date are summarized below:

- TIRC7 protein structure is predicted as a seven transmembrane protein
- Cell surface expression of TIRC7 is induced in antigen-primed T cells as an early event following antigen-induced T cell activation
- TIRC7 is expressed primarily on activated T cells, most notably in $CD4^+CD28^+$ effector T cell subsets
- TIRC7 expression occurs to be induced on B cells

Enhanced immune responses observed in TIRC7 null mice summarized below provide a solid basis for its negative regulatory roles:

- Increased T cell responses and cytokine production upon various stimulation
- Increased B cell responses and Ig production upon various stimulation
- Enhanced DTH responses *in vivo*
- Increased levels of serum Ig in all classes

Anti-TIRC7 antibodies act as agonists and induce inhibitory effects in T cells by:

- Suppressing antigen-induced T cell proliferation
- Suppressing production of Th1 cytokines, including IL-2 and IFN-γ
- Inhibiting IL-2 receptor expression at transcriptional level
- Inducing expression of negative regulator CTLA4
- Inhibitory effects of TIRC7 pathway are mediated, at least in part, by CTLA4

$TIRC7^+$ cells are found in human tissues with various diseases where T cell activation plays a key pathogenic role:

- Including RA, MS, DTH and rejection of transplanted organs

Treatment with anti-TIRC7 mAb, which cross-reacts with murine and rat TIRC7, demonstrated marked therapeutic effects in the following pre-clinical disease models:

- In the collagen-induced arthritis (CIA) mouse model for RA:

  Efficacy was observed not only in the disease prevention model, but also in the therapeutic model with established arthritis

- In experimental autoimmune encephalitis (EAE) model for MS:

  Antibody therapy halted disease progression of established EAE

- In organ transplant models (mouse cardiac allograft model and rat kidney allograft model):

  Induction of donor-specific anergy was observed, resulting in a significant prolongation of graft survival, including long-term graft survival

  Up-regulation of CTLA4 and down-regulation of CD25 were induced in the infiltrating cells

  Synergy with calcineurin inhibitors was observed, resulting in prolonged graft survival at doses that would be ineffective as monotherapy

The HLA DR alpha 2 domain was identified as the ligand protein TIRC7 and functional studies using the soluble HLA DR alpha 2 domain revealed

- inhibition of lymphocyte proliferation
- Th1 cytokine expression

The results obtained utilizing either TIRC7 antibodies or soluble ligand HLA DR alpha 2 domain in various assays not only contribute to a basic understanding of lymphocyte stimulation, but might also have substantial clinical implications to develop novel therapeutic and diagnostic approaches for the treatment of undesired immune responses.

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ABBREVIATIONS

7-TM	Seven transmembrane
Ab	Antibody
APC	Antigen presenting cells
CII	Type II collagen
CIA	Collagen-induced arthritis
CsA	Cyclosporine A
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DMARDs	Disease modifying anti-rheumatic drugs
DTH	Delayed type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
i.m.	Intramuscular injection
i.p.	Intraperitoneal injection
i.v.	Intravenous injection
mAb	Monoclonal antibody
MLR	Mixed lymphocyte reaction
MMC	Mitomycin
MS	Multiple sclerosis
MTX	Methotrexate
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
RA	Rheumatoid arthritis
SQ	Subcutaneous injection
TIRC7	T cell immune response cDNA 7
TNFR	Tumor necrosis factor receptor
UC	Ulcerative colitis

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ERKLÄRUNG

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, daß

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wird bzw. wurde,
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