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TIRC7 Deficiency Causes In Vitro and In Vivo Augmentation of T and B Cell Activation and Cytokine Response

Nalán Utku,* Anke Boerner,† Antje Tomschegg,† Fatima Bennai-Sanfourche,† Grit-Carsta Bulwin,‡ Thomas Heinemann,‡ Jürgen Loehler,§ Richard S. Blumberg,¶ and Hans-Dieter Volk*

The membrane protein T cell immune response cDNA 7 (TIRC7), in human T lymphocytes and demonstrated that TIRC7 exhibit a central role in T cell activation (1). TIRC7 represents a novel protein involved in immune regulation, as it does not share structural or sequence homology with any of the known molecules involved in lymphocyte functions (1). Similar types of seven-membrane proteins have recently been characterized as important regulators of the immune response, including CCR, which belong to the group of G-coupled protein receptors (2). Chemokine receptors are involved in the regulation of a number of inflammatory conditions and serve as important targets for development of therapeutics for the treatment of immune-mediated diseases (3). For example, it has been shown that CCR1-deficient mice are completely resistant to the development of experimental allergic encephalomyelitis, and CCR5-deficient mice exhibited decreased CD62L, but increased CD11a and CD44 expression, suggesting an in vivo expansion of memory/effector T cells. Remarkably, activated T cells from TIRC7−/− mice expressed significantly increased proliferation and expression of IL-2, IFN-γ, and IL-4 in response to different stimuli. Resting T cells from TIRC7−/− mice exhibited increased CD62L, but decreased CD11a and CD44 expression, suggesting an in vivo expansion of memory/effector T cells. Interestingly, activated T cells from TIRC7−/− mice expressed lower levels of CTLA-4 in comparison with wild-type cells. B cells from TIRC7-deficient mice exhibited significantly higher in vitro proliferation following stimulation with anti-CD40 Ab or LPS plus IL-4. B cell hyperreactivity was reflected in vivo by elevated serum levels of various Ig classes and higher CD86 expression on B cells. Furthermore, TIRC7 deficiency resulted in an augmented delayed-type hypersensitivity response that was also reflected in increased mononuclear infiltration in the skin obtained from TIRC7-deficient mice fed foods. In summary, the data strongly support an important role for TIRC7 in regulating both T and B cell responses. The Journal of Immunology, 2004, 173: 2342–2352.

We recently identified the seven-span transmembrane protein, T cell immune response cDNA 7 (TIRC7), in human T lymphocytes and demonstrated that TIRC7 exhibit a central role in T cell activation (1). TIRC7 represents a novel protein involved in immune regulation, as it does not share structural or sequence homology with any of the known molecules involved in lymphocyte functions (1). Similar types of seven-membrane proteins have recently been characterized as important regulators of the immune response, including CCR, which belong to the group of G-coupled protein receptors (2). Chemokine receptors are involved in the regulation of a number of inflammatory conditions and serve as important targets for development of therapeutics for the treatment of immune-mediated diseases (3). For example, it has been shown that CCR1-deficient mice are completely resistant to the development of experimental allergic encephalomyelitis, and CCR5-deficient mice exhibit resistance to the development of experimental colitis.

The TIRC7 gene is localized on chromosome 11q13. The gene expresses TIRC7 and a splice variant, OC116, by tissue-specific alternative splicing (4). OC116, also named atp6, is solely expressed in osteoclasts (5, 6). Although the functional role of OC116 remains to be defined, mutations of the gene encoding OC116 and TIRC7 have been shown to be associated with the development of autosomal recessive infantile malignant osteopetrosis in humans (7–11).

Other proteins have also been described to exhibit a functional role in osteoclasts as well as T and B cell immune response such as TNFR-associated factor 6 and TNF family molecules osteoprotegerin (12, 13). Mice disrupted for the osteoprotegerin gene (receptor activator of NF-κB ligand) exhibit severe osteopetrosis and a defect in tooth eruption, lack of osteoclastogenesis, as well as defects in early T and B cell development (14). TNFR-associated factor 6-deficient mice were also shown to exhibit osteopetrosis and impaired B cell proliferation (13).

Targeting of TIRC7 with specific Abs causes inhibition of Th1 (IL-2 and IFN-γ), but not Th2 (IL-4), cytokine secretion in human lymphocytes in association with a prolonged state of T cell unresponsiveness upon alloantigen and mitogen activation in vitro (1). In an in vivo model of acute kidney allograft rejection in rats, administration of anti-TIRC7 Abs results in a marked prolongation of allograft survival. Furthermore, we have recently demonstrated that treatment with a mAb against TIRC7 in an allotransplanted mouse heart model significantly prolongs allograft survival and induces CTLA-4 expression in mononuclear cells infiltrating the transplanted heart tissue (15).

To learn more about the role of TIRC7 in immunoregulation, we generated mice deficient in TIRC7 expression using homologous recombination. The results obtained from the present study demonstrate that TIRC7 plays an important regulatory function in both T and B cell responses.

Materials and Methods

Construction of TIRC7-deficient mice

Gene targeting was performed into C57BL/6 mice in accordance with established protocols (16). In embryonic stem (ES) cells derived from strain 129SvJ mouse embryos, a 2- kb genomic fragment of exons 2–8 of TIRC7

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2 Abbreviations used in this paper: TIRC7, T cell immune response cDNA 7; ES, embryonic stem; DAB, diaminobenzidine; DTH, delayed-type hypersensitivity; PALS, periarteriolar lymphocyte sheath; PNA, peanut agglutinin; WT, wild type.

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was replaced by insertion of a cassette containing the neomycin resistance gene. Transfection and culturing of the ES cells were performed, as previously described (17). Heterozygous animals were interbred to generate homozygous animals, and offspring were examined by PCR. Samples were subjected to 2.5% agarose gel electrophoresis using TAE running buffer (0.04 mol/L Tris acetate, 0.001 mol/L EDTA).

Flow cytometric analysis

Cells were stained for 30 min at 4°C in 100 μl of PBS and then washed before analysis. FACS analysis was performed, as previously described (18). Cells were stained with a panel of fluorochrome-conjugated Abs, including FITC-labeled anti-CD3 mAb and anti-Gr-1 mAb, PE-labeled anti-CTLA-4, anti-CD3, anti-CD25, anti-CD69, anti-CD44, anti-CD62L, anti-CD11a, anti-CD71, anti-CD28, and anti-CD86 Abs. PerCP-labeled anti-B220 and anti-CD4 and allophycocyanin-labeled anti-CD4 mAb and anti-CD8 mAb were purchased from BD Pharmingen (San Diego, CA). For intracellular staining, cells were permeabilized for 10 min with hyperperm solution. Analyses were performed using a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). Cells were analyzed using CellQuest software (BD Biosciences). For TIRC7 binding on splenocytes, cells were blocked with 10% mouse serum for 1 h and stained with FITC-labeled anti-TIRC7 mAb (GenPat77) or FITC-labeled control Ab (BD Pharmingen).

Histological analysis and immunohistochemistry

Tissue specimens were fixed with Bouin's solution or 4% formaldehyde containing 1% acetic acid and embedded in Paraplast X-TRA (Sherwood Medical, St. Louis, MO). Deparaffinized sections were stained with H&E, periodic acid-Schiff reaction, and modified Giemsa-Wright stain (all obtained from Sigma-Aldrich, Taufkirchen, Germany), according to standard protocols. To immunohistochemically demonstrate Ig L and H chains, TCR, myeloperoxidase, lysozyme, cathepsin, and inducible NO synthase by an indirect immunoperoxidase technique, a panel of polyclonal Abs was used: rabbit anti-mouse κ and L chains (Bionetics, Newport News, VA), goat anti-mouse μ H chain (Pel-Freez, Rogers, Arizona), rabbit anti-mouse H chain (Jackson-Dianova, Hamburg, Germany), rabbit anti-mouse α H chain (Nordic, Tilburg, The Netherlands), rabbit anti-human CD3 (DAKO Diagnostika, Hamburg, Germany), rabbit anti-human myeloperoxidase (DAKO Diagnostika), rabbit anti-inducible NO synthase (Affinity BioReagents, Golden, CO), rabbit anti-lysozyme (Biogenesis, Poole, U.K.), and rabbit anti-cathepsin D (Linaris, Bad Nauheim, Germany). For Ag retrieval, deparaffinized sections were treated with a commercial target unmasking fluid (DAKO Diagnostika) at 98°C for 20 min in a microwave oven. Incubation with primary Abs was performed overnight at 4°C. Specifically bound primary Abs were detected using a highly sensitive peroxidase- and polymer-conjugated anti-rabbit Ig detection system (Envision; DAKO Diagnostika, Hamburg, Germany). Peroxidase activity was evaluated with the diaminobenzidine (DAB) or the aminoethylcarbazol technique (DAB plus and aminoethylcarbazol plus substrate kits from DAKO Diagnostika). Finally, sections were counterstained with hemalum and permanently coverslipped. For the simultaneous demonstration of T and B cells, a double labeling technique was used. Briefly, deparaffinized sections were incubated overnight with an Ab mixture containing appropriately diluted rabbit anti-human CD3 (DAKO Diagnostika) and rat anti-mouse CD45R (B220) (BD Pharmingen) mAbs. Subsequently, specifically bound rabbit and rat Abs were detected separately by first incubating the sections with the peroxidase- and polymer-conjugated anti-rabbit Ig detection system (Envision; DAKO Diagnostika), followed by DAB visualization of peroxidase activity, and second, by overlaying the sections with biotinylated goat anti-rat Ig Abs (Rat Link; BioGenex, San Ramon, CA), followed by phosphatase-labeled streptavidin (Super Sensitive System; BioGenex), which was visualized thereafter by the Vector Blue substrate kit (Vector Laboratories, Burlingame, CA). Binding of peanut agglutinin (PNA) to germlinal center cells was revealed by incubating sections for 60 min with 30 mg/ml PNA (Sigma-Aldrich) and subsequent reactions with rabbit anti-PNA Abs (DAKO Diagnostika), and peroxidase- and polymer-conjugated anti-rabbit Ig detection system (Envision; DAKO Diagnostika), followed by DAB visualization of peroxidase activity (19). Endogenous avidin-binding activity was reduced by pretreatment of the sections with avidin and biotin solutions (Zymed Laboratories, San Francisco, CA).

Morphological analysis of apoptosis was performed by using spleen tissue and staining with H&E for detection of apoptotic bodies around the central splenic artery of wild-type (WT) and knockout mice spleens using a 30× objective. To identify the apoptosis index for each spleen, each tissue sample was subjected 10 times to microscopic analysis of apoptotic bodies.

For enumerating apoptotic lymphocytes, H&E-stained paraffin sections of the spleens of five TIRC7−/− and six age- and sex-matched WT animals were used. Typical apoptotic bodies were counted at ×1000 on a microscope (Axiostar; Zeiss, Göttingen, Germany) using an eyepiece equipped with a grid for reticulocyte counts (Zeiss). As determined by an objective micrometer scale (Zeiss), the area of the larger square of the grid measured 0.0064 mm² at ×1000. In each section, the number of apoptotic lymphocytes was counted in at least 10 random sites of the splenic periarteriolar lymphocyte sheets (PALS). The final values were calculated per mm² of splenic PALS tissue.

Preparation of cells from spleens

Single cell suspensions of mouse spleens were prepared by grinding tissue through a sterile wire mesh and passing through a 50-μm filter. All procedures were conducted under sterile conditions in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% FCS (Biochrom), 5 mM glutamine, penicillin, and streptomycin (Invitrogen Life Technologies, Eggenstein, Germany).

Cell proliferation assays

For T cell proliferation assays, lymphocytes were stimulated with 10 μg/ml anti-CD3, anti-CD28 mAb (BD Pharmingen) in precoated wells, or PHA (1 or 2 μg/ml) (Sigma-Aldrich) at 37°C for 48 h in 96-well plates. Cultures were then pulsed with 1 μCi of [3H]thymidine (Valeant Pharmaceuticals, Costa Mesa, CA), and after 18-h incubation, cells were harvested and cell proliferation was determined by measuring thymidine incorporation (cpm) using a scintillation counter. For B cell proliferation assays, lymphocytes (2 × 10⁶ cells/ml) were stimulated with 10 ng/ml IL-4 and either 0.5 μg/ml anti-CD40 mAb (BD Pharmingen) or 100 ng/ml LPS (Sigma-Aldrich) for 48 h. Cells were pulsed with 1 μCi of [3H]thymidine, and cell proliferation was measured after 18 h. Levels of IgM and IgG were measured in supernatants of 7-day-old cultures by ELISA using capture, detection, and standard Abs obtained from BD Pharmingen.

Cytokine production of lymphocytes

A total of 2 × 10⁶ cells/ml of lymphocyte suspensions (from mouse spleens) was incubated with 1.5 μg/ml PHA or 100 ng/ml LPS and 10 ng/ml IL-4 (BD Pharmingen) at 37°C for 48 h in microtiter plates. IFN-γ, IL-4, and IL-2 cytokines were measured in supernatants of PHA-stimulated cultures. Cytokine levels were determined by ELISA using capture, detection, and standard Abs obtained from BD Pharmingen.

Serum concentrations of Ig isotypes

Blood was obtained from the retro-orbital plexus of mice. The serum concentrations of IgM, IgG1, IgG2a, IgG2b, IgG3, IgA, and IgE were determined by ELISA using capture, detection, and standard Abs obtained from BD Pharmingen.

Delayed-type hypersensitivity (DTH) assay

The DTH response to OVA (Sigma-Aldrich) was estimated by footpad swelling, as previously described (20). Briefly, mice were sensitized with an intraperitoneal injection of 50 μl of 5% (w/v) OVA emulsified in CFA (Sigma-Aldrich) at the base of the tail. Eight days after the initial immunization, mice were challenged with an injection of 30 μl of 2% (w/v) OVA in PBS into the left planar footpad and 30 μl of PBS alone in the right planar footpad. Footpad thickness (swelling and erythema) was measured in both footpads, and the magnitude of the DTH reaction was determined as the difference in footpad thickness between OVA- and PBS-injected footpads. Plantar skin obtained from the injected footpads was removed, fixed in 10% (w/v) buffered formalin, embedded in paraffin, sectioned, and stained with H&E for microscopic evaluation.

Results

Generation of TIRC7-deficient mice

To analyze the role of TIRC7 in immune responses, we generated mice in which the TIRC7 locus was disrupted by gene targeting using homologous recombination (16). A targeting vector was constructed and used to replace sequences encoding exons 2–8 of the TIRC7 gene with the neomycin drug resistance gene (Fig. 1A). Genotyping of the progeny resulting from an intercross of two animals heterozygous for the disrupted TIRC7 gene locus was performed by PCR analysis using TIRC7-specific oligonucleotide primers (Fig. 1B) and revealed the expected three types of offspring in a Mendelian frequency. The lack of the TIRC7 protein on CD3-positive T lymphocytes isolated from homozygous offspring

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FIGURE 1. A. Homologous recombination was performed in 129 SvJ ES cells using a vector construct containing the neomycin resistance gene to replace exons 2–8 of the TIRC7 gene. Heterozygous mice were generated by injection of ES cells into C57BL/6 blastocysts. Primer location for PCR genotyping was chosen within the deleted WT sequence (P1), the neomycin cassette (P3), and nondeleted 3' region (P2), and resulted in the amplification of a 1.2-kb WT fragment and a 1.4-kb mutant fragment of TIRC7 alleles.

B. Offsprings of an intercross of heterozygous mice were genotyped by PCR analysis of the genomic DNA and revealed WT (+/+), heterozygous (+/−), and homozygous (−/−) mice with regard to the disrupted TIRC7 gene locus, as demonstrated in PCR amplification products on an agarose gel (n = 70).

C. To demonstrate the lack of TIRC7 expression in 48-h PHA-activated splenocytes of TIRC7-deficient (−/−) and WT littermate mice (+/+), confocal microscopic analysis was performed using (Figure legend continues)
was confirmed by staining with an anti-TIRC7 Ab and subsequent confocal microscopy (Fig. 1C). The gross phenotype of TIRC7-deficient mice revealed a significant reduction in body weight. TIRC7-deficient mice reached only 30% of the body weight of WT mice and exhibited relatively pronounced head size at the time of birth and subsequent retardation of postnatal growth in comparison with WT littermates (Fig. 1D).

**TIRC7-deficient mice exhibit a decrease of mononuclear cell populations and severe atrophy of primary and secondary lymphoid organs**

Flow cytometric analysis of single cell suspensions of splenocytes stained with cell lineage-specific Abs revealed markedly decreased numbers of lymphocytes in TIRC7-deficient mice. Splenic T lymphocytes were reduced by ∼50% and splenic B lymphocytes by 60% relative to the respective numbers in WT animals (Fig. 1E).

The ratio of CD4 and CD8 T cells was not significantly altered. However, a marked increase in the number of granulocytes was observed in the cell suspensions. These findings were reflected by the results of the histological analysis that revealed atrophy of the primary and secondary lymphoid organs in TIRC7-deficient mice. As shown in Fig. 2, prominent hypoplasia of the splenic white pulp with disorganized T and B cell areas was observed in the spleens of TIRC7-deficient animals (Fig. 2A) compared with WT littermates (Fig. 2B). The structural alterations in the splenic white pulp of TIRC7-deficient mice were characterized by numerous relatively large PALS and small B lymphocyte follicles representing dramatically diminished B cell areas (Fig. 2C) in comparison with WT littermates (Fig. 2D) as well as increased numbers of germinal centers in the B lymphocyte follicles. But despite this finding, the numbers of germinal centers in the B follicles were increased. In contrast to the hypoplasia observed in the splenic white pulp of

**FIGURE 2.** Shown are spleen sections of 3-wk-old TIRC7-deficient mice (A, C, E, and G) and WT littermates (B, D, F, and H). A and B. Using Giemsa-Wright staining, the white pulp of TIRC7/−/− mice (A) was found to be hypo- and dysplastic due to an irregularly developed B cell corona around the PALS compared with WT littermates (B). The arrowhead points to an abnormally positioned germinal center that lacks a lymphocytic mantle zone. The red pulp contains increased numbers of granulocytes and megakaryocytes. C and D, CD3 (brown) and B220 (blue) immunohistochemical double labeling revealed a relatively expanded PALS and greatly reduced B cell zone in the splenic white pulp of TIRC7 mutants (C). The normal distribution pattern of the B (blue) and T cell (brown) compartments in the splenic white pulp of WT animals is depicted in D. E and F. Immunostaining for κ and λ L chains demonstrated a pronounced plasma cell hyperplasia in spleens of TIRC7/−/− mutant (E) compared with WT littermates (F). G and H. Using myeloperoxidase immunostaining, hyperplasia of the granulocyte cell lineage in the red pulp of TIRC7/−/− mutants (G) became visible compared with myeloperoxidase-positive granulocytic cells in the splenic red pulp of WT littermates (H). Magnification (A–H) ×145. The results represent one representative example of four experiments.
TIRC7-deficient animals, splenic red pulp was hyperplastic due to greatly increased granulopoiesis and megakaryocytopenia, as demonstrated by cell lineage-specific immunostaining (Fig. 2G), compared with WT animals (Fig. 2H). In the splenic red pulp of TIRC7-deficient mice, a prominent plasma cell hyperplasia was observed (Fig. 2E) compared with WT mice (Fig. 2F), as revealed by Ig L chain immunolabeling. Likewise, an increased percentage of CD3-positive T cells was noted to invade the red pulp, although the total T cell numbers were decreased in the atrophic spleen. Histological analysis of the thymus obtained from TIRC7-deficient mice revealed different stages of atrophy with masses of apoptotic thymocytes primarily observed in the cortex (data not shown). The morphological configuration of peripheral and mesenteric lymph nodes was unchanged, but appeared paucicellular compared with the WT animals (data not shown). In the paracortex, the numbers of lymphoblasts and of lymphocytes undergoing apoptosis were increased. In the splenic PALS of TIRC7-deficient mice, we observed a trend toward an increased number of apoptotic bodies (67.8 ± 76.3 per mm² of splenic white pulp tissue, n = 5) in comparison with WT mice (26 ± 7.7 per mm² of splenic white pulp tissue, n = 6). The presence of perivascular cuffs of lymphocytes and lymphocytes invading the wall of high endothelial venules in the paracortex of lymph nodes indicated that a normal recirculation activity was present in TIRC7-deficient animals. Obviously abnormal, however, were accumulations of granulocytes in the medullary cords. In the Peyer’s patches of the intestines of TIRC7-deficient animals, only a relative reduction of the B cell areas and paucicellularity was observed. The liver exhibited increased numbers of T cells and plasma cells compared with the WT mice, as assessed by CD3 and Ig L chain immunostaining (data not shown). Similar alterations of lymphoid organs, particularly those of the splenic white pulp, were already observed in newborns (24 h after birth), suggesting that the alterations are genetically determined rather than the result of abnormal adaptive immune reactions.

**Alloantigen or mitogen activation in cells lacking TIRC7 leads to a significant increase in T cell proliferative responses and induction of Th1- and Th2-type cytokines**

Marked alterations were also observed in the activation state of lymphocytes isolated from TIRC7-deficient mice, as assessed by the incorporation of [³H]thymidine into nuclear DNA. Stimulation of TIRC7-deficient splenocytes with anti-CD3 Ab either alone or in combination with an anti-CD28 Ab resulted in a significantly increased proliferative response in comparison with that observed in WT mice (Fig. 3A). Similarly, following mitogenic activation of splenocytes with PHA, a dose-dependent increase in the proliferative response of splenocytes isolated from TIRC7-deficient mice was observed that substantially exceeded that observed in cells from WT animals (Fig. 3B). Furthermore, using ELISA, we

**FIGURE 3.** A. Proliferation was determined in splenocytes isolated from TIRC7-deficient (−/−) and WT (+/+ ) mice by [³H]thymidine uptake. Cells were activated for 48 h with a, either medium alone (med), anti-CD3 mAb alone (αCD3), or in combination with anti-CD28 mAb (αCD3, αCD28), or b, PHA at different concentrations. B. The production of IFN-γ, IL-2, and IL-4 was determined by ELISA in the supernatants of nonstimulated cells in medium (med) or PHA-stimulated splenocytes from WT (+/+) and TIRC7-deficient mice (−/−) 48 h after culturing. Each bar represents the mean of data obtained from three animals.
determined the levels of cytokines expressed by splenocytes in the culture supernatants 48 h after PHA stimulation. In TIRC7-deficient splenocytes, the levels of IFN-γ, IL-2, and IL-4 were increased in comparison with the levels detected in the supernatants from WT splenocytes (Fig. 3D). Similar results were obtained with lymphocytes isolated from lymph nodes (data not shown). Thus, these results are consistent with a pronounced hyperresponsiveness of TIRC7-deficient Th1 and Th2 lymphocytes as a consequence of alloantigen and mitogen stimulation.

**TIRC7 deletion affects expression of activation-associated surface molecules on T cells**

To assess the role of TIRC7 in the overall process of T lymphocyte stimulation, we examined the expression of several molecules known to be involved in lymphocyte activation and costimulation. CD69 and CD25, both representing marker molecules for activated T cells, were detected at moderately increased levels in nonstimulated T lymphocytes from TIRC7-deficient mice compared with WT mice. CD62L, a marker for naive T cell populations, was slightly diminished in TIRC7-deficient lymphocytes, suggesting an activated phenotype. Consistent with this, CD44, which represents a marker for activated and memory T cells, was elevated on TIRC7-deficient cells in comparison with WT cells (Fig. 4A). Effector and memory T cells are also notable for expression of high levels of CD11a (LFA-1) (18). The percentage of CD11a high cells was observed to be increased in TIRC7-deficient T cells in comparison with WT lymphocytes (19 vs 4%) (Fig. 4B). In comparison, the CD11a low population of T cells was diminished in TIRC7-deficient lymphocytes (81 vs 96%). When lymphocytes were activated with PHA for 48 h, the proportion of cells exhibiting high CD11a expression was also much more pronounced in TIRC7-deficient mice in comparison with WT lymphocytes (56 vs 28%) (Fig. 4B). These studies indicate that although TIRC7 deficiency results in a pronounced decrease in overall T cell numbers, the cells remaining are mainly consistent with activated effector/memory cells.

**TIRC7 deletion affects expression of several surface molecules of T cells**

In a separate series of studies, we examined the expression of several costimulatory molecules, including CTLA-4, CD40L, and CD28. CTLA-4 is a central component of a negative costimulatory pathway during T cell activation. Upon engagement of CD28 with B7 molecules on the surface of APCs, intracellular CTLA-4 is mobilized to the cell surface of lymphocytes, where it competes, at higher affinity, with CD28 for binding to B7-1 (CD80) and B7-2 (CD86) molecules (21–24). The intracellular and cell surface expression of CTLA-4 was determined to be permeabilized as well as nonpermeabilized lymphocytes by FACS analysis 48 h after PHA activation (18). Although CTLA-4 expression was up-regulated on WT lymphocytes in a regular manner 48 h after activation, PHA activation of lymphocytes isolated from TIRC7-deficient mice resulted in markedly reduced intracellular and cell surface expression of CTLA-4 (Fig. 4C). In contrast, no differences were observed for either CD28 or CD40L expression on resting and activated T cells from TIRC7-deficient in comparison with WT mice (Fig. 4D). The relatively low expression of CD28 on splenocytes obtained from WT and TIRC7 knockout mice might be explained by the young age of these mice compared with the higher expression levels of CD28 as reported for splenocytes of 6- to 9-wk-old mice (25). To determine whether the expression of other receptors on the cell surface was affected by the deficiency of TIRC7, we analyzed the expression of the transferrin receptor (CD71), which is known to undergo endocytosis and recycling. As shown in Fig. 4D, the levels of CD71 were not altered by the absence of TIRC7 expression (Fig. 4D). The latter results indicate that TIRC7 deficiency does not exhibit a general effect on cell surface molecules involved in recycling. The results suggest a link between TIRC7-mediated signals and CTLA-4 expression on T lymphocytes.

**Deletion of TIRC7 results in increased B cell activation and elevation of Ig levels**

To determine whether the effect of TIRC7 deficiency is limited to T lymphocytes or also affects B lymphocytes, the in vitro proliferation of splenocytes was examined following a 48-h incubation with B cell-stimulating agents, specifically anti-CD40 Ab alone and LPS in combination with IL-4. As shown in Fig. 5A, the proliferative response of TIRC7-deficient splenocytes under these conditions, as assessed by [3H]thymidine incorporation, was substantially increased following activation of B cells with both stimuli in comparison with WT splenocytes. These types of stimuli were also accompanied by a marked increase in the secretion of IgM and IgG1 Abs into the culture supernatants of in vitro stimulated B cells (Fig. 5B). Differences were also observed in Ig levels in vivo in TIRC7-deficient mice. In TIRC7-deficient mice, serum levels of Ig classes, especially IgM, IgG1, and IgG2a, were observed to be increased at various levels, as determined by ELISA (Fig. 5C). These results suggest that TIRC7 deficiency also results in excess B lymphocyte activation, which is substantiated by the above-mentioned morphological observations. To further prove this point, the expression of molecules involved in B lymphocyte stimulation was examined in splenocytes stimulated with LPS and IL-4. As shown in Fig. 5D, B220-positive resting B lymphocytes obtained from TIRC7-deficient mice exhibited higher levels of CD86 expression on the cell surface in comparison with WT littermates. In contrast, no significant differences were observed in the expression of CD80 on B cells from TIRC7-deficient mice in comparison with WT littermates (data not shown). These results indicate that TIRC7 deficiency also affects the expression of cell surface molecules involved in the ability of B cells to function as APCs.

**Mice lacking TIRC7 are more responsive to Ag in vivo**

The studies described above suggested that TIRC7 deficiency results in a state of lymphocyte hyperresponsiveness in vivo. On the basis of these results, we examined the effects of TIRC7 deficiency in an in vivo model of a DTH response. Mice were sensitized to OVA by intradermal injection into the footpad and rechallenged 8 days after immunization. The severity of the immune reaction was assessed by determining the circumference of the resulting footpad swelling (20). Footpad swelling peaked at 48 h after Ag rechallenge in both control and TIRC7-deficient mice. However, the magnitude of the swelling observed in the TIRC7-deficient mice was substantially greater than that observed in the WT littermates (38% TIRC7+/− vs 17% WT). Histological analysis of the skin obtained from the injected footpads revealed a significantly increased mononuclear and granulocyte infiltration in the TIRC7-deficient mice in comparison with the WT littermates, consistent with a much more severe degree of inflammation in the TIRC7-deficient group (Fig. 6). These results confirmed the in vivo relevance of the lymphocyte hyperresponsiveness in the absence of TIRC7 expression and revealed that, in the context of TIRC7 deficiency, increased numbers of granulocytes are observed upon Ag activation despite a paucicellularity of T and B lymphocytes.
Discussion
TIRC7 is a recently described seven-span transmembrane protein that was identified by differential display RT-PCR from mRNA obtained from activated vs resting T cells. TIRC7 exhibits strong homology between different mammalian species. Targeting TIRC7 with polyclonal Abs recognizing peptides obtained from the putative extracellular loops has been previously shown to cause strong inhibition of T cell proliferation and Th1 cytokine secretion following mitogen and alloantigen stimulation in vitro as well as abolish acute allograft rejection in vivo (1, 15).

To analyze more directly the role of TIRC7 in immune regulation, we generated TIRC7-deficient mice by homologous recombination. In this study, we provide further evidence that TIRC7 plays an essential role in the regulation of immune responses. We analyzed several immune functions in vitro and in vivo in TIRC7-deficient mice in comparison with their WT littermates.
Overall, 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. Targeting of the TIRC7 molecule with polyclonal Abs was previously shown to inhibit both proliferation and Th1 cytokine secretion (1). This effect required cross-linking of TIRC7, as Fab did not mediate the inhibitory effects (N. Utku, unpublished observation). On the basis of the previously published data, it was not possible to determine...
whether TIRC7 is a stimulatory or inhibitory molecule because the Abs used could have either triggered a negative signal provided by TIRC7 or blocked a positive signal induced by unknown physiological TIRC7 ligand(s). The enhanced T cell proliferation and Th1 cytokine secretion observed in TIRC7-deficient mice following different T cell stimuli suggest that TIRC7 normally induces negative effects on T cell activation. In contrast to our previous report that TIRC7 specifically regulates Th1 cytokine secretion in human lymphocytes (1), the examination of TIRC7-deficient mice revealed that in the absence of TIRC7, both Th1 and Th2 cytokine responses are affected. At the simplest level, the difference observed between rodent and human T cells might be species related. It might also reflect a threshold effect, with Th1 responses being more sensitive than Th2 responses. It is also possible that different agonistic signals delivered by TIRC7 as modeled by Ab-mediated ligation may differentially regulate Th1 vs Th2 cytokine secretion. In this regard, we have observed that other Abs we have generated in our laboratory and that are currently under investigation also have inhibitory effects on Th2 responses in rodent models. Obviously, the deletion of the entire TIRC7 gene product in the current report affected Th1 and Th2 response, suggesting that TIRC7 differentially regulate both Th1 and Th2 cytokine expression. Determining the molecular basis for these effects is worthy of future investigation.

Consistent with the T cell hyperactivity observed in vitro, we observed significantly higher levels of memory/effector T cells in 2-wk-old TIRC7-deficient mice. In accordance with this, T lymphocytes from TIRC7-deficient mice expressed lower levels of CD62L and higher levels of CD11a and CD44. Indeed, the in vivo response to an exogenous Ag, OVA, as assessed in a DTH response model, was significantly increased in TIRC7-deficient mice in comparison with WT littermates.

In addition to immune abnormalities, TIRC7-deficient mice exhibited significantly lower birth weight and a retardation of postnatal growth. The latter finding is most likely due to abnormalities in bone metabolism related to the diminished expression of a splice product of the TIRC7 gene, OC-116, in osteoclasts (4, 26). Previous studies have shown that OC-116 knockout mice exhibit bone abnormalities and retardation of postnatal growth. However, it is notable that in these animals, all organs, including immune-related organs, were reported to be functionally and histologically normal (26). This finding is most likely due to continued expression of TIRC7 in OC116 knockout mice, as the OC116 deficiency was generated by deletion of exons 2–5, which are specific for the OC116 gene product (Fig. 7A). In contrast, TIRC7 deficiency as generated in the current study leads to deletion of both OC116 and TIRC7, because the deleted exons 2–8 are shared by both proteins (Fig. 7A). Our histopathological findings of bone structure in TIRC7-deficient mice provide evidence for severe osteopetrosis, as described in OC116-deficient mice. Based upon the previously described phenotype of OC116-deficient mice (26), we can conclude that the abnormal immune function and histopathological findings observed in the TIRC7-deficient mice are most likely due to the deficiency of the TIRC7 protein and not due to the growth retardation related to the loss of OC116 expression (Fig. 7B). Also, it is likely that the gross morphologic changes observed in the TIRC7-deficient mice were related to concomitant deletion of the OC116 gene product (26).

Although the mechanism by which TIRC7 regulates immune responses is unknown, our data allow for some conclusions. In B cells, TIRC7 deficiency caused hyperreactivity, as shown by enhanced proliferation, Ig synthesis, and CD86 expression following LPS and IL-4 stimulation in vitro as well as elevated serum levels of most Ig classes and increased numbers of germinal centers and
plasma cells in vivo. The finding of B cell hyperresponsiveness in the absence of TIRC7 expression is consistent with preliminary data from our laboratory suggesting that TIRC7 is also expressed on B cells (N. Utku, unpublished observations). This suggests that, under normal circumstances, TIRC7 may provide negative regulatory functions in B cells through signaling pathways that are to be defined.

In T cells, it was found that TIRC7 deficiency caused a diminished up-regulation of CTLA-4 following T cell activation in vitro compared with normal T lymphocytes. Both membrane and intracellular levels of CTLA-4 were reduced before and after T cell activation in TIRC7-deficient mice. CTLA-4 is a key molecule that controls T cell responses and is up-regulated within 24–48 h following T cell stimulation. CTLA-4 interacts with the ligands of the constitutively expressed costimulatory molecule CD28, B7-1 (CD80), or B7-2 (CD86), on APCs. In contrast to CD28, CTLA-4 interactions mediate inhibitory signals for the activated T cell, preventing overstimulation. CTLA-4-deficient mice were shown to exhibit hyperproliferation and autoimmunity early after birth and die from uncontrolled immune reactivity (27). Although the in vitro and in vivo responsiveness of immune cells from TIRC7−/− mice exhibit some similarities to CTLA-4-deficient mice, the phenotypes are not completely comparable. In contrast to CTLA-4−/− mice, we observed no significant enhancement of spontaneous T cell proliferation in vitro and much less evidence for hyperplasia of secondary immune organs and enhanced autoimmunity in vivo. The less severe phenotype in TIRC7-deficient mice might be explained by a residual CTLA-4 expression in TIRC7−/− mice. However, it is likely that other than CTLA-4-mediated regulatory pathways are triggered by TIRC7 as well. Like CTLA-4, TIRC7 is weakly expressed on resting T cells and up-regulated within 24 h following T cell stimulation (1). It is now well established that molecules involved in T cell activation are not randomly distributed over the cell surface, but form an immune synapse around the TCR/CD3 complex (28). Interestingly, TIRC7 colocalizes with the TCR, CTLA-4, and other molecules of the synapse as detected by confocal microscopy (N. Utku, unpublished observations). Moreover, TIRC7 is detectable within clathrin-coated vesicles like other molecules associated with the immune synapse (N. Utku, unpublished observations). These observations suggest that TIRC7 is directly involved in controlling the immune response as a component of the immune synapse perhaps independently and/or in addition to its effects upon CTLA-4 expression and function.

Overall, the results described in the current study provide strong support for the notion that TIRC7 is involved in the regulation of receptor molecules that are essential for a balanced T and B lymphocyte-mediated immune response. Our previous studies, using Ab-mediated ligation as a model, suggest that TIRC7 ligation mediates the induction of lymphocyte hyporesponsiveness. Consistent with this, TIRC7 deficiency leads to lymphocyte hyperresponsiveness. As such, these studies contribute not only to a basic understanding of lymphocyte costimulation, but also may have substantial clinical implications because targeting of TIRC7 to specifically induce hyporesponsiveness in lymphocytes may be a therapeutic modality for transplantation surgery and the treatment of autoimmune diseases.

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**FIGURE 7.** A, Genomic organization of the exon-intron boundaries of OC116 and TIRC7. Arrows indicate the deletions of exons that were constructed to generate the knockout mice for TIRC7 and OC116, respectively. B, Summary of the phenotypes of TIRC7 and OC116 knockout mice.
References


