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Antibody targeting of TIRC7 results in significant therapeutic effects on collagen-induced arthritis in mice

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Summary

TIRC7 is a cell surface molecule which is expressed in T and B lymphocytes and negatively regulates their function. Anti-TIRC7 specific monoclonal antibody (mAb) inhibited T cell memory response to recall antigens. Up-regulation of TIRC7 on lymphocytes from joint tissue of patients with Rheumatoid Arthritis (RA) and mice with collagen induced arthritis (CIA) suggested TIRC7 as a novel target to promote anti-inflammatory reaction. Anti-TIRC7 mAb administration significantly inhibited the induction and progression of CIA and the anti-collagen IgG1 and IgG2a antibody response. Combination therapy of anti-TIRC7 mAb and soluble TNF-α receptor demonstrated an increased inhibitory effect over the single compounds on CIA. The results demonstrate the therapeutic potential of TIRC7 targeting with mAb in diseases associated with exaggerated T and B cell responses.

Keywords: antibody targeting, TIRC7, collagen-induced arthritis

Introduction

Many autoimmune diseases are associated primarily with an exaggerated Th1 response [1,2]. 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 significant efforts to understand the underlying pathogenetic mechanisms. A lack of clarity with regard to both the predisposing factors and the precise antigenic targets of the immune response have restricted the development of effective therapeutic approaches [2]. Recent data suggest a significant contribution of B cells to the pathogenesis of many autoimmune diseases in addition to the (auto)antibody production of these cells [3–5]. These findings suggest the need for specific modulation of both T- and B-cells for sufficient treatment of autoimmune diseases. An excellent example to illustrate this need is RA, a common human autoimmune disease characterized by a chronic inflammatory reaction in the synovium of joints which is associated with cartilage degeneration and juxta-articular bone erosion [2]. The histopathological features of synovitis in RA involve massive leucocyte infiltration consisting primarily of macrophages and CD4+ T lymphocytes but also of B cells [3]. The conventional therapy and the more recent selective anti-inflammatory therapy targeting TNF-α and IL-1 interact with late effector mechanisms of the disease resulting in high efficacy but limited long-term success. Moreover, long-term neutralization of effector cytokines compromises anti-infectious response [2].

The mouse model of collagen-induced arthritis (CIA) is the most appropriate small animal model available for human RA and was shown to be particularly advantageous for the study of the T cell dependent erosive arthritis, as it mimics the symptoms seen in human RA [2,6]. This model has not only been instructive in understanding the role of T cells in RA pathogenesis but also led to a recent appreciation that B cells, through their secretion of IgG and the deposition of complement components, play a critical role in regulating the induction and effector phase of synovial infiltration and joint destruction in arthritis [6,7]. Although differences exist between human RA and CIA in mice, the CIA mouse model has been successfully used to analyse effects of compounds such as TNF-α receptor blockers for their therapeutic potential for the treatment of human RA [8].

TIRC7, a novel T and B cell membrane molecule, has been shown to play an important role in immune activation [9–13]. TIRC7 is up-regulated within few hours after T-cell stimulation, and intragraft detection of TIRC7 predicts acute heart [12] and kidney allograft rejection episodes. Targeting of TIRC7 with anti-TIRC7 specific antibodies has been shown to be effective in inhibiting T cell proliferation and Th1 cytokine expression *in vitro* as well as in preventing organ allograft rejection *in vivo* [9,12]. Recent data suggests the mechanism of anti-TIRC7 mAb-mediated immune

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modulation is via the delivery of a negative signal to T cells via TIRC7. Targeting of TIRC7 with mAb is associated with an up-regulation of CTLA4, an important negative regulator of T cell function [12], *in vitro* and *in vivo* which was shown to be decreased in TIRC7 deficient mice [13]. However, TIRC7 deficient mice exhibit immune hyperactivity of both T and B cells, suggesting a role of TIRC7 in regulation of both lymphocyte subsets [13]. The option of targeting T- and B-cell activation in parallel makes TIRC7 a novel candidate for effectively combating autoimmune diseases associated with T- and B-cell dysregulation.

The present study was conducted to examine the effects of TIRC7 targeting on T- and B-cell function and the therapeutic potential of a monoclonal antibody (mAb) against TIRC7 either alone or in combination with soluble TNF-α receptor in collagen-induced arthritis (CIA) in mice.

Methods

Monoclonal antibody generation and characterization

Female BALB/c mice (Charles River Laboratory, Sulzfeld, Germany) were immunized with TIRC7 protein and fusion of spleen with myeloma cells and antibody identification was performed and mAb was tested on TIRC7 transfected COS7 cells as described in Utku *et al.* [9].

Induction of DTH

Female BALB/c mice (Charles River) were sensitized by a subcutaneous injection of 5% Ovalbumin (chicken egg, Sigma, Deisenhofen, Germany) emulsified with complete Freund's adjuvant (cFA, Sigma) into the base of the tail. After eight days, the mice were challenged by an injection of 2% heat-denatured OVA in physiological solution into the left plantar footpad. The right plantar footpad received physiological solution as a control. Footpad swelling was measured using a dial caliper (Mitutoyo Corp., Tokyo, Japan) 24 and 48 h after the challenge. The magnitude of the DTH response was determined as the difference in footpad thickness between OVA- and physiological solution-injected footpads. BALB/c mice ($n = 7$) received anti-TIRC7 mAb or control mAb ($n = 7$) 500 μg/day starting on day 0, 0·5 h prior to and 2 h after the administration of the antigen, followed by 500 μg on day 1–6 intraperitoneally (i.p).

For the induction of DTH with oxazolone, BALB/c mice were injected i.p. with 500 μg of either anti-TIRC7 or control mAb. Twenty hours after mAb treatment mice were presensitized by painting 150 μl of the haptenating agent, oxazolone (4-ethoxymethylene-2-phenly 2-oxazolin-5-one; Sigma-Aldrich), 3% dissolved in 100% ethanol onto a shaved abdomen. Five days after presensitation, 1% oxazolone in 20 μl of 100% ethanol or ethanol alone as control was painted on the right and left ears, respectively. Ear swelling was measured before and 24 h after the ear challenge with a dial thickness gauge (Mititoyo, Kanagawa, Japan). DTH responses were expressed as the increase in ear swelling after oxazolone painting on the ear following subtraction of the thickness before the challenge for the control and experimental group. A fragment of the centre portion of the ear from six mice in each group was assessed after paraffin embedding by standard haematoxylin and eosin (H&E) staining, and three sections from each block were examined.

Histopathology

Plantar footpad skin or center portion of the ear samples of hind footpads were excised, fixed in 4% buffered formalin, embedded in paraffin, sectioned and stained with H&E using standard techniques. For immunohistochemical staining, formalin-fixed paraffin-embedded samples (5 μm) were deparaffinized and rehydrated according to standard protocols. Heat-assisted antigen retrieval was performed in a microwave, and slides were heated in MW-buffer (DAKO, Germany).

Sections were blocked in 5% milk/PBS and incubated with a rabbit polyclonal anti-TIRC7 antibody (10) in a dilution of 1 : 25, for 12 h at 4°C. After washing, slides were incubated with Cy3-conjugated anti-rabbit antibody (1 : 250, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. As a negative control, normal rabbit IgG (500 μg/ml, Santa Cruz) was used. The stained sections were examined by confocal laserscan microscopy (Axiovert 100 M, Carl Zeiss, Göttingen, Germany). Synovial fluid was obtained during the course of therapeutic arthrocentesis from patients with RA of the knee and collected in sterile tubes containing ACD. Mononuclear cells from synovial fluid samples were separated under sterile conditions by Ficoll-Paque density gradient and cryopreserved in enriched medium prior to long-term storage in liquid nitrogen. Human samples were obtained as slides from Oligene GmbH, Berlin, Germany.

Induction of collagen induced arthritis in mice (CIA)

Eight week-old male DBA/1 mice (Charles River Laboratory) were immunized intradermally at the base of the tail with 100 μg bovine collagen type II (CII, Sigma) emulsified in complete Freund's adjuvant (cFA, Sigma). Mice were rechallenged with CII in incomplete Freund's adjuvant (iFA, Sigma) 21 days later. CIA development was assessed daily for signs of joint inflammation of the four paws and was graded by the following score: 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. The maximal arthritis score per paw was 4 and the maximal disease score per mouse was 16 [14,15].

Radiological and histological examination of joints

Fore and hind paws were radiographed with a X-ray imaging system in cooperation with the Veterinary Institute of Pathology, Free University of Berlin. For each animal the metacarpal-phalangeal joints with digits 2–5, carpal-metacarpal joint, metatarsal-phalangeal joints with digits 1–5 and the tarsal-metatarsal joints were assessed. Radiographs were examined for erosions of cartilage and bone structure, osteolysis, disfiguration and curvatures. Joints with lesion were counted and a cumulative X-ray score was attained for each animal. Histopathological analysis was performed as described above. Microscopic evaluation of arthritic paws was carried out by an individual who did not know which treatments the animals had received. Arthritic changes in the carpus, tarsus, metacarpophalangeal, metatarsophalangeal and proximal interphalangeal joints were examined for inflammation and joint damage. A lesion severity score of 0–4 was assigned to each paw. Thus, an accumulative score of 0–16 was possible for each animal [15].

Flow cytometry analysis

Peripheral blood mononuclear cells (PBMC) obtained from patients after informed consent and approval from the Ethics Committee of the Charite Hospital, Berlin, or blood from mice were stained for 30 min at 4 °C in PBS and then washed prior to analysis. We used a panel of fluorochrome-conjugated antibodies, including FITC-labelled anti-CD3 mAb and anti-CD19 mAb, anti-CD16 mAb, anti-CD3 mAb, PerCP-labelled anti-CD4 mAb and APC-labelled anti-CD4 mAb and anti-CD8 mAb. (Pharmingen, Germany). Gates were set on the lymphocyte population. Analyses were performed using a FACSCalibur (BD, Germany). Cells were analysed using Cell Quest software (Becton Dickinson).

Proliferation, cytokine and IgG ELISA assays

PBMCs from healthy donors were isolated by Ficoll-Hypaque density centrifugation using standard methods. PBMCs were stimulated with alloantigen or recall antigen mixture of CMV (kindly provided by Dr V.S. Baehr, Institute of Immunology, Charite, Berlin), Candida (Alyostal), Influenza (Begrivac) and Tetanus (Merieux) incubated for 48 or 96 h, respectively, at 5% CO_2, 37 °C in the presence of anti-TIRC7 mAb and IgG-control. Spleen mononuclear cells from mice were isolated using standard methods. 10^4 cells in a final volume of 0·1 ml of RPMI 1640 medium supplemented with 10% fetal calf serum, 1 mM L-glutamine and streptomycin-penicillin were added to individual wells of 96-well microtitre plates. Mononuclear cells were stimulated by incubation with PHA (1·5 μg/ml) or LPS (200 ng/ml) at 5% CO_2, 37 °C for 48 h. After 48 h incubation-time 1 μCi ^{3}H-thymidine was added per well and the plates were incubated for a further 14–18 h at 37 °C, 5% CO_2 in an incubator. Cells (96-well plates) were harvested and transferred to NC-plates using a harvester. Ninety-six-well plates were allowed to dry for 4 h at room temperature and then the back sides of the nitrocellulose filters were covered with a seal. To transform the radioactive signal produced by the samples into fluorescence, 30 μl of scintillation fluid was added and counts per minute were measured using a beta-counter (Perkin Elmer Life Sciences). Supernatants were collected after 48 h of culture and cytokine levels quantified by sandwich ELISA (Cytokine Kits, BD). The production of IFN-γ, IL-2, and IL-10 was measured in the supernatants of mononuclear spleen cells of mice 48 h after PHA stimulation and of TNF-α and IL-6 48 h after LPS stimulation. Cytokine levels were determined by ELISA using antibodies from Pharmingen.

Results

Anti-TIRC7 mAb inhibits memory T cell response *in vitro* and *in vivo*

To examine the functional role of TIRC7 expression on memory T cells we analysed the effects of TIRC7 ligation with an anti-TIRC7 mAb [12,13], which was raised against human TIRC7 and shown to cross-react with mouse TIRC7 protein, on the proliferative response to recall antigen of human PBMC. A mixture of several recall antigens (tetanus toxoid, influenza, candida, CMV) was used to ensure a potential response from all study subjects. As shown in Fig. 1, in the presence of soluble anti-TIRC7 mAb a significant inhibition of proliferation was observed at approximately 12.5 μg/ml mAb. When the mAb was coated to the culture plate overnight, the inhibitory dose of the mAb required to inhibit the proliferation was much lower which is

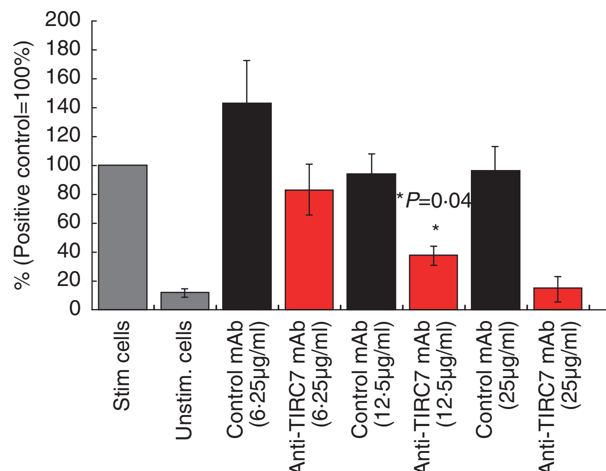


Fig. 1. Anti-TIRC7 mAb treatment inhibits memory T cell response. Human PBMC were exposed to anti-TIRC7 mAb in the presence and absence of recall antigens such as CMV, Influenza, Pertussis Toxin and Candida which resulted in a significant down regulation of immune response ($P = 0·04$). Shown is ± SD of IC50 obtained from three independent experiments.

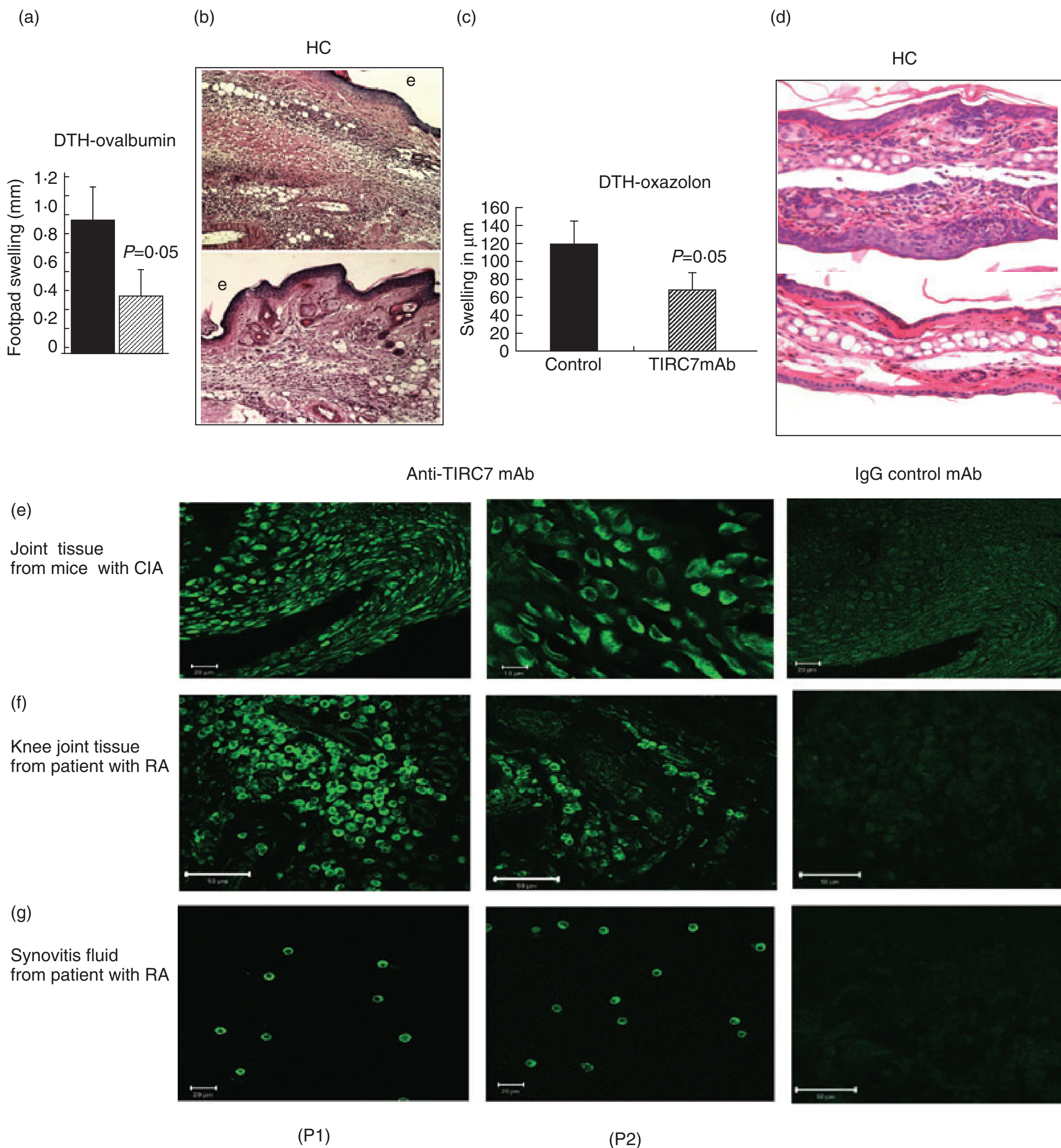
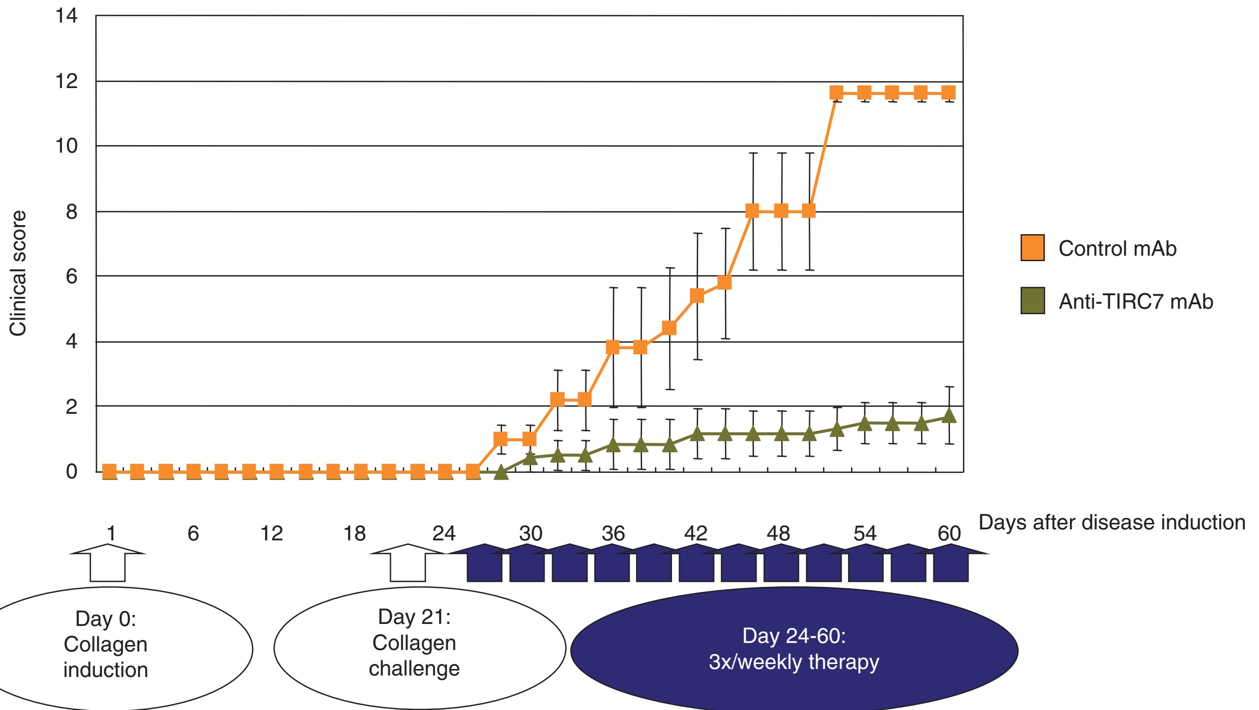


Fig. 2. TIRC7 is highly expressed in infiltrates of joints and anti-TIRC7 mAb inhibits T cell response. (a) Mean footpad swelling of the anti-TIRC7 antibody treated mice ($n = 7$) (▨) was inhibited 48 h after the second challenge with Ovalbumin compared to control mAb treated mice (■). (b) A histochemistry analysis (HC). One example out of seven is shown. (c) Mean ear swelling of oxazolone sensitized mice treated with the anti-TIRC7 antibody prior to skin painting ($n = 10$) (▨) was significantly inhibited after the challenge with oxazolone compared to control mAb treated mice (■). (d) A histochemistry analysis (HC). Administration of the anti-TIRC7 mAb inhibited leucocyte infiltration into ear skin during both DTH responses. Severe infiltration of lymphocytes was observed in the control mAb treated group compared to minimal infiltration in the anti-TIRC7 mAb treatment group. One example out of three is shown. (e–g) Anti-TIRC7 mAb staining revealed up-regulation of TIRC7 protein expression in tissues obtained from knee joints of DBA mice and mice with established CIA (e, animal 1 (P1) and animal 2 (P2))(two examples out of three are shown). TIRC7 expression was also observed in knee joint samples (f, middle panels, patient 1 (P1) and patient 2 (P2)) (two examples out of five are shown), and in mononuclear cells from the synovial fluid (g, lower panels, patient 1 (P1) and patient 2 (P2)), obtained from patients with established RA (two examples out of five are shown). No staining was observed in all experiments using control mAb for staining (right panel, e–g).

in support of an agonistic action of the mAb binding to TIRC7 (data not shown). In separate proliferation assays using PHA, Fab fragments of the anti-TIRC7 mAb were not able to inhibit proliferation (data not shown) which support the hypothesis of an agonistic mAb action.

Furthermore, in a delayed type hypersensitivity (DTH) model, mice were immunized in the footpad with ovalbumin (OVA) in complete Freund's adjuvant (cFA) and treated with either anti-TIRC7 mAb or control mAb every day for seven days (Fig. 2). We used the anti-human anti-TIRC7 mAb

(a)



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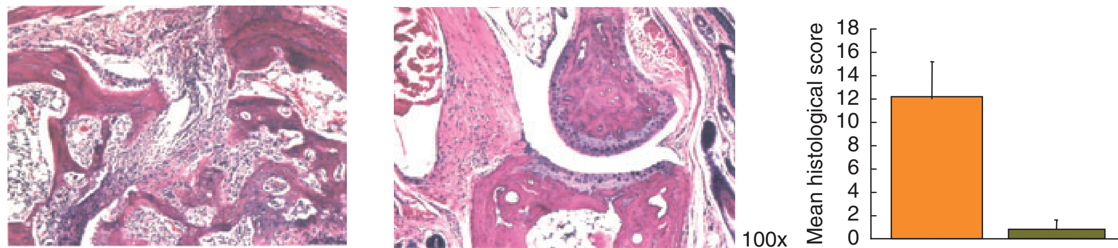

(c)

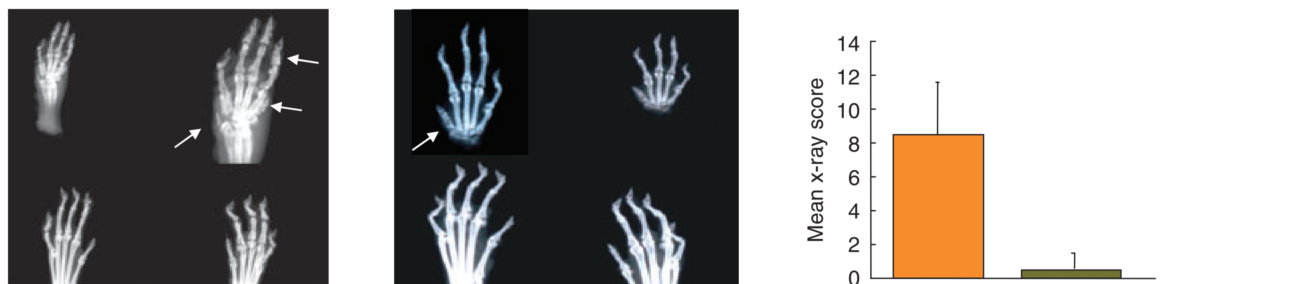

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Fig. 3. Anti-TIRC7 mAb treatment significantly prevents progression of CIA in mice. (a) Anti-TIRC7 mAb provides a preventive effect after a second challenge with collagen in mice. DBA/1 mice were treated with 500 μg anti-TIRC7 mAb or control mAb three times per week starting on day 24. No clinical symptoms were observed at the time of the treatment initiation. Mice in the control mAb group ($n = 6$) developed typical clinical symptoms of moderate to severe 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 during the course of the study compared with control animals, and the maximal mean arthritis score reached by the end of the study was 2·5 (green curve). (b) The therapeutic effect of an anti-TIRC7 mAb treatment after a second challenge with collagen starting at day 24 is confirmed by histology. Evaluation at low magnification revealed 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 mAb treated mice (left, arrow). In contrast, there was no histological abnormality recorded in the joint microarchitecture of anti-TIRC7 mAb mice (right). This is also reflected in the mean histological score of anti-TIRC7 mAb treated animals (■) in comparison to control animals (▨). (c) A significant therapeutic effect of anti-TIRC7 mAb treatment after second challenge was also confirmed by X-ray studies. A radiograph examination of a control mouse showed signs of arthritis with severe erosions on the joint surface, disfiguration and osteolysis (left, arrow). A radiograph from a mouse treated with the therapeutic anti-TIRC7 mAb demonstrated neither lesions nor evidence of joint damage, bone loss or disfiguration (right, arrow). This was also reflected in the mean X-ray score of anti-TIRC7 treated animals (■) in comparison to control animals (▨).

which was demonstrated to cross react with mouse TIRC7 *in vitro* and followed the same administration regimen *in vivo* as was demonstrated to be effective in preventing cardiac transplant rejection in mice [12]. Footpad swelling was compared between the anti-TIRC7 and control mAb treated groups. These studies revealed significantly reduced swelling of the footpads in anti-TIRC7 mAb treated animals in comparison with control animals (Fig. 2a). Animals treated with the anti-TIRC7 mAb also exhibited significantly reduced mononuclear cell infiltration in comparison to the controls (Fig. 2b). This inhibition of the delayed type hypersensitivity reaction (DTH) was also observed after a single administration of the anti-TIRC7 mAb dose at the time of DTH induction using oxazolone as a haptenizing reagent. Anti-TIRC7 mAb treatment showed a significant prevention of ear swelling of mice in comparison to control mAb treated mice. These results were reflected in the histological analysis of the skin of mice as infiltration of mononuclear cells was prevented with the treatment of the mAb against TIRC7 (Fig. 2c,d).

TIRC7 expression is up-regulated in lymphocytes of knee joint lesions and synovial fluid obtained from patients with rheumatoid arthritis

Following cardiac allotransplantation we demonstrated an up-regulation of TIRC7 in intragraft lymphocytes, whereas TIRC7 was described to be down-regulated in PBMC of the same animals [13]. Based on these results we wondered whether TIRC7 might also be up-regulated in inflammatory immune diseases of different etiology such as RA. Connective tissue of the knee joint of five patients with established RA and five patients with Osteoarthritis (OA) was examined by immunohistopathology and correlated with the respective clinical phenotypes. In addition, lymphocytes from the synovial fluid were obtained from five additional patients with established RA and subjected to immunostaining and subsequent confocal microscopic analysis using a direct FITC conjugated anti-TIRC7 mAb. As expected, the knee joint tissues of patients with active RA contained a marked mononuclear infiltration. Moreover, the infiltrating lymphocytes exhibited significant TIRC7 staining (Fig. 2f). In addition, synovial fluid lymphocytes from patients with established RA also exhibited significant levels of TIRC7 expression (Fig. 2g). In contrast, in joint tissues obtained from patients with OA no TIRC7 expression was observed (data not shown). The same applied for tissues of control mice without arthritis. Since CIA in mice is the most approximate model of human RA available in small animals and widely used we performed the same examination in mice with established CIA. Again, while control animals without CIA did not show any TIRC7 staining (not shown) a significant up-regulation of TIRC7 was observed in lymphocytes infiltrating the joint tissue of CIA mice (Fig. 2e).

Anti-TIRC7 mAb prevents the progression of collagen-induced arthritis

As anti-TIRC7 antibody treatment prevents allograft rejection, we were interested in examining whether anti-TIRC7 mAb therapy prevents the progression of autoimmune disease.

CIA was induced with collagen and after 21 days a second collagen induction was performed. Three days after the second collagen induction, animals were treated for about 5 weeks with either the anti-TIRC7 mAb or control mAb (500 μg/injection) three times per week. By this regimen, anti-TIRC7 mAb treatment yielded a significantly lower mean arthritis score, reaching only 2·5 at the end of the study at day 60 in comparison to the control group which yielded a mean arthritis score of 12 (Fig. 3a). Histological (Fig. 3b) and radiological analysis (Fig. 3c) supported these clinical observations by showing a significant reduction in disease activity in the group receiving TIRC7 mAb.

Anti-TIRC7 mAb exhibits a significant therapeutic effect in established arthritis either alone or in combination with recombinant soluble TNF-α receptor protein

The development of novel therapeutics for the treatment of undesired immune activation like RA focuses on various

different mechanisms of the immune response. Current clinical treatment of RA involves the administration of human soluble TNF-α receptor Fc fusion protein (TNF-R:Fc) which binds to soluble TNF-α thereby preventing the signalling pathway mediated by TNF-α [15,16]. A significant number of patients with RA, however, do not show a sufficient response to treatment with TNF-α blocking agents. As TIRC7 mAb was demonstrated to prevent the onset of CIA, we were interested to examine whether anti-TIRC7 mAb has therapeutic effects in established inflammatory disease.

We administered anti-TIRC7 mAb either as monotherapy or in combination with soluble TNF-α receptor after reaching a clinical score 1 in the CIA mouse model. The human TNF-α receptor protein was reported to cross react with murine TNF [17]. After animals reached a clinical CIA score of 1, groups of animals were treated with either anti-TIRC7 mAb ($n = 7$), control mAb ($n = 7$), TNF-α receptor construct ($n = 7$), or combination of both, TNF-α receptor and anti-TIRC7 mAb ($n = 7$). During the first seven days of the study, animals were treated every day using either 1000 μg/day, i.p., in the monotherapy group with anti-TIRC7 mAb, or 50 μg/day, i.p., in the TNF-α receptor treatment group, or half of each compound in the combination therapy group, followed by administration of the respective compound three times per week (Fig. 4a). By the end of the study period (day 24 post treatment) mice treated with the anti-TIRC7 mAb alone exhibited a significantly lower mean clinical arthritis score (mean < 6) in comparison to control antibody treated mice (mean ~ 11) (Fig. 4a). The group treated with TNF-R:Fc alone demonstrated a mean clinical score of 7. The combination of anti-TIRC7 mAb and TNF-R:Fc resulted in a significant reduction of clinical arthritis symptoms with a mean clinical score of about 3·3. Histological (Fig. 4b) and radiological (Fig. 4c) findings were consistent with the respective arthritis score and showed a significantly increased clinical effect of anti-TIRC7 mAb in combination with TNF-R:Fc compared with monotherapy of either compound. In fact, only one animal out of seven in the combination group showed progression of arthritis symptoms.

To assess whether the clinical benefit of each treatment was associated with changes in lymphocyte function, all animals were analysed for their ability to respond to antigens as measured by cytokine secretion and immunoglobulin expression patterns. All treatment groups demonstrated normal proliferative responses to collagen, mitogens and LPS (data not shown). The groups treated with anti-TIRC7 mAb alone revealed decreased levels of IFN-γ compared with the control group and unchanged levels of TNF-α, IL-4, and IL-10 secretion (Fig. 4d,e). Animals treated with TNF-R:Fc alone showed significantly decreased levels of TNF-α (Fig. 4d). However, IFN-γ (Fig. 4d), IL-4, and IL-10 remained unchanged (Fig. 4e). The combination of anti-TIRC7 mAb with TNF-R:Fc resulted in decreased levels of IFN-γ and TNF-α, and unchanged levels of IL-4 and IL-10 secretion (Figs 4d,e) These results suggest an additive effect of both compounds.

Antibody targeting of TIRC7 alone or in combination with soluble TNF-R:Fc regulates B cell responses *in vivo*

CIA development has been shown to be dependent upon both, cellular and humoral immune responses to collagen II [3]. To determine whether treatment with the anti-TIRC7 mAb, TNF R:Fc, or a combination of both, altered the humoral response in the CIA model, we measured concentrations of anti-collagen IgG1 and anti-collagen IgG2a antibodies in sera of mice in the various treatment groups. Anti-collagen antibodies were assayed at the end of the treatment period. Control antibody treated mice exhibited severe clinical CIA which was associated with elevated total anti-collagen IgG1 and IgG2a antibodies (Fig. 4f). Animals treated with anti-TIRC7 mAb alone or in combination with soluble TNF-R:Fc exhibited a profound blunting of the anti-collagen IgG1 and anti-collagen IgG2a secretion which was significantly reduced compared with that in CIA mice receiving control mAb (Fig. 4f). Administration of TNF-R:Fc alone caused a significant reduction of anti-collagen IgG2a secretion but only mild reduction of IgG1. These results suggest that anti-TIRC7 mAb treatment prevents disease progression in CIA through effects on the T cell as well as the B cell response in the CIA model in mice.

Discussion

CIA is a murine model of CD4-positive T cell dependent erosive arthritis that is used to study immune mechanisms and pathways which are important in human RA, a predominantly Th1-mediated disease. It has also recently become recognized that B cells through their secretion of complement fixing antibodies also contribute significantly to the development of arthritis in animal models of RA [3–5]. Furthermore, the fact that B cell deficiency also provides protection against the development of severe CIA points towards an independent role of complement components associated with the alternative pathway of activation as being important in disease pathogenesis [18].

Anti-CD20 mAb targeting B cells [19] in RA as well as CTLA4-Ig targeting of T cell response demonstrate favourable results in clinical studies [20]. Based on these results it can be hypothesized that a novel therapy modulating both T- and B-cell response might offer alternate beneficial treatment for patients suffering from this disease. As was recently demonstrated by our group, targeting of TIRC7 with specific mAb resulted in a strong inhibition of T cell proliferation *in vitro* [9,12]. These results are further supported by our observation made in the present study that treatment of mice with the anti-TIRC7 mAb prevented a DTH reaction. In T cells, TIRC7 is predominantly up-regulated on CD4 and CD8-positive memory T cells. Compared with intracellular

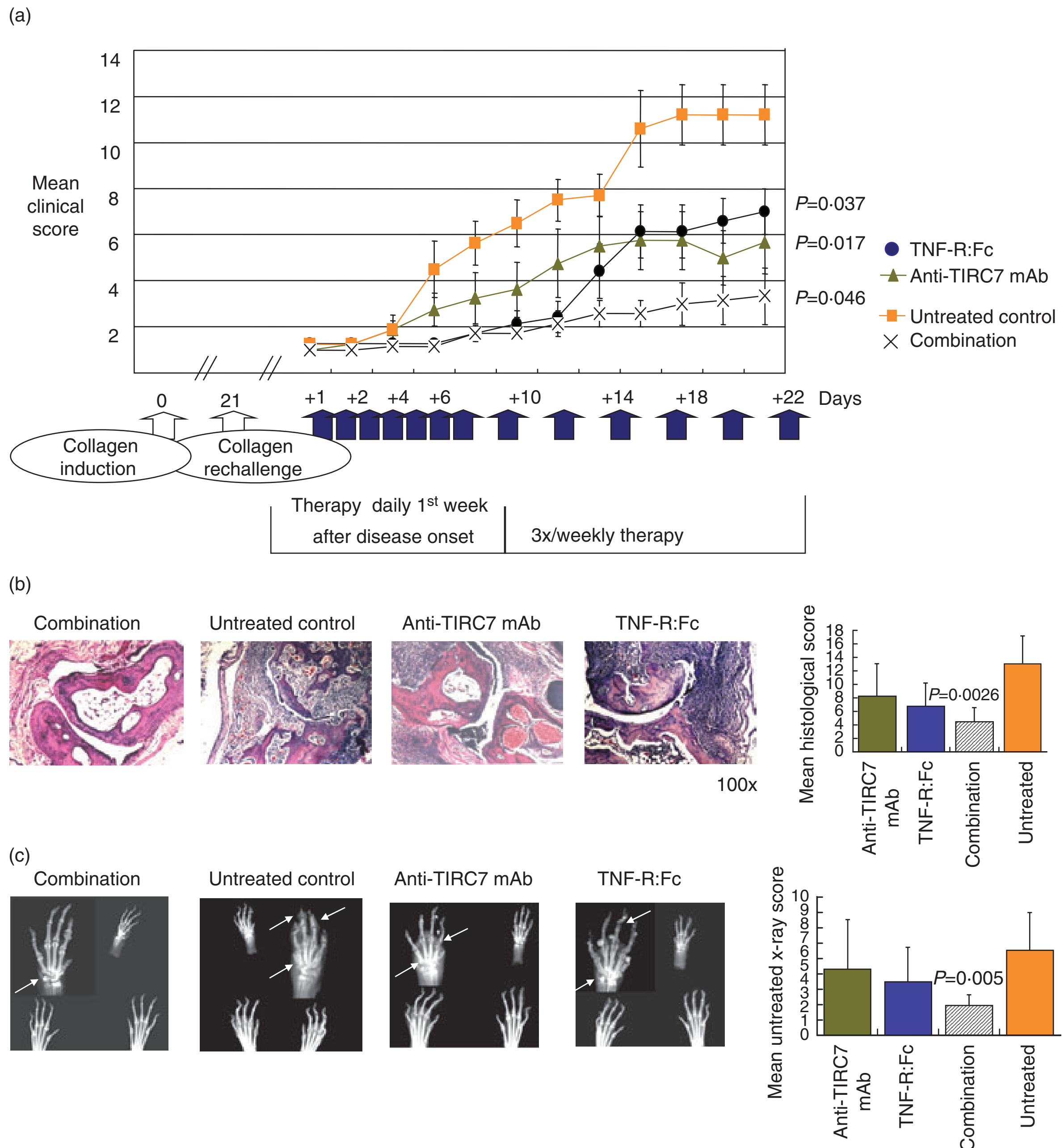

Fig. 4. Anti-TIRC7 mAb as monotherapy or in combination with soluble TNF-α fusion protein is highly effective in established CIA in mice. (a) Either an anti-TIRC7 mAb alone (1000 μg/day) or TNF-α receptor: Fc (50 μg/day) alone or anti-TIRC7 mAb (500 μg/day) in combination with soluble TNF-α receptor: Fc (25 μg/day) (Enbrel®) was given i.p. at the day when mice reached a clinical score of 1 for two weeks (3-weekly therapy). Mice treated with anti-TIRC7 antibody showed a mean clinical score of 6 (green curve), whereas TNF-α receptor: Fc treated group exhibited a mean clinical score of 7 and the combination group a score of 3 after two weeks of treatment. The control mAb group showed a mean score of 10 (orange curve). Only animals which developed a score of 1 within 7 days after the second collagen induction were included in the study and randomly distributed to the various groups. Animals which did not reached a score of 1 within 7 days after second challenge were not included in any experimental group. (b, c) The effects of anti-TIRC7 mAb or soluble TNF-α receptor: Fc alone or combination of both compounds are confirmed by histology (b) and X-ray analysis (c) of all joints. (d, e) Anti-TIRC7 antibody or combination with soluble TNF-α receptor: Fc caused a selective effect on cytokines. Treatment with anti-TIRC7 mAb for five weeks (green bar) did not affect the expression of TNF-α, IL-4, and IL-10 in splenocytes whereas IFN-γ is significantly ($P = 0{\cdot}05$) reduced in comparison to the control mAb treated group (orange bars). Administration of TNF-α receptor: Fc construct (blue bar) resulted in a significant reduction of TNF-α levels ($P = 0{\cdot}02$) whereas no changes were observed in the expression of IL-4, IL-10, and IFN-γ. The combination of anti-TIRC7 mAb with soluble TNF-α receptor: Fc revealed a reduction of IFN-γ and TNF-α (hatched bar). (f) Anti-TIRC7 mAb alone or in combination with soluble TNF-α receptor: Fc reduced anti-collagen antibody responses in sera of mice with established CIA. Compared to control mice (orange bars) the production of anti-collagen antibodies IgG1 and IgG2 was reduced whereas soluble TNF-α alone revealed no changes for the IgG1 levels.

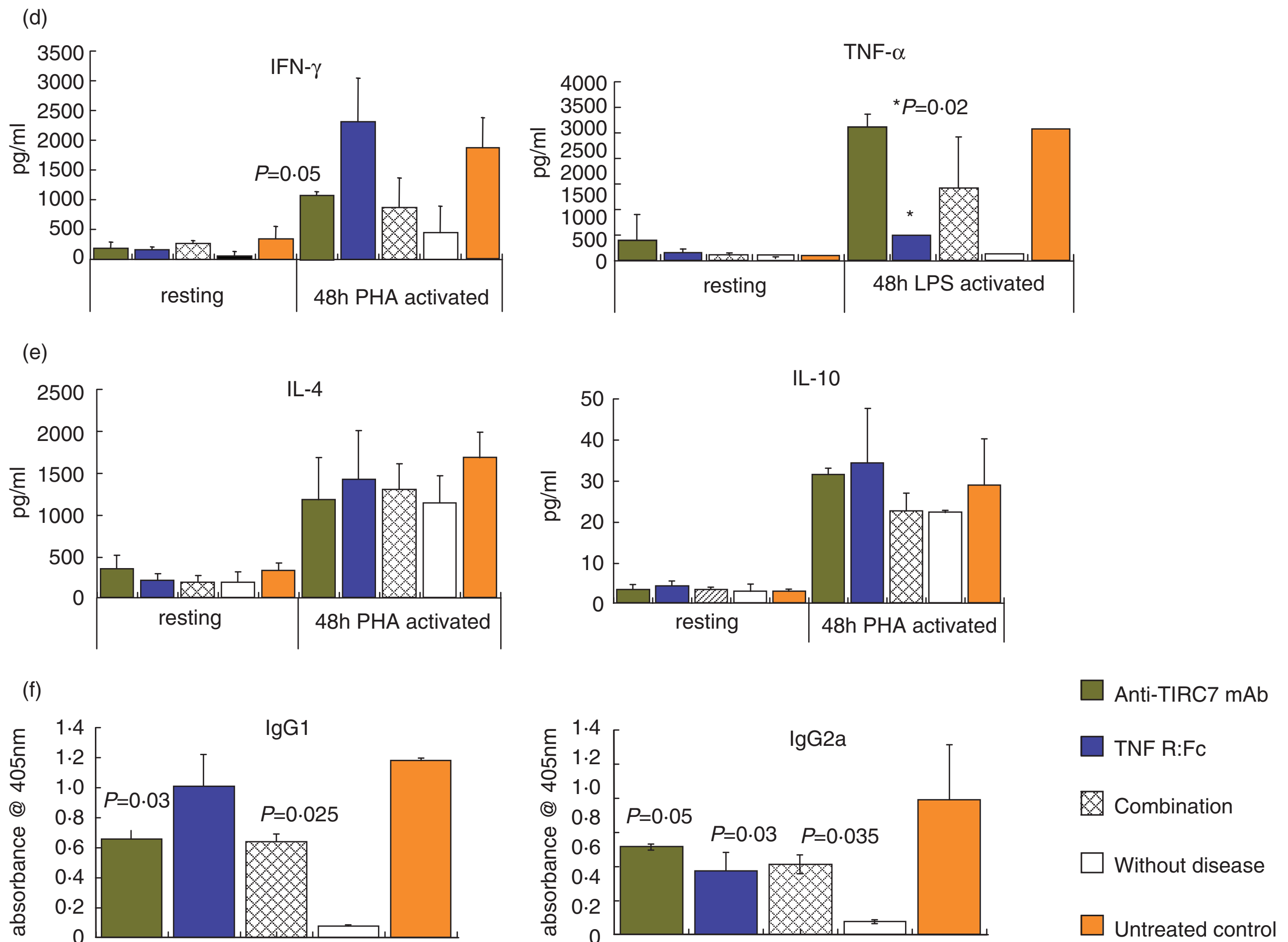

Fig. 4. *Continued*

levels of TIRC7 the surface expression of TIRC7 is relatively low in T cells (unpublished observations). This expression profile of TIRC7 with a 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. The inhibition of lymphocyte responses depends on the function of a variety of molecules such as CTLA4 [20]. In previous studies we demonstrated an up-regulation of CTLA4 in lymphocytes in the presence of anti-TIRC7 mAb resulting in inhibition of proliferation [12]. In addition, anti-TIRC7 mAb does not exhibit antiproliferative effects on CTLA4 deficient splenocytes suggesting that TIRC7 negative signalling depends predominantly on CTLA4 in T cells. These data support the view that TIRC7 mAb activates an inhibitory pathway via the CTLA4 protein (unpublished observation).

In addition, in the present study, we demonstrate that 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 which showed elevated cytokine secretion and increased immune activation, suggesting lack of an inhibitory influence on lymphocyte activation [13]. The results of a down-regulation of both classes of antibody responses rather than a specific effect on only IgG2a responses suggest an independent 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.

TNF-α was shown to be effective in treatment of arthritis in a CIA mice model [8]. It was also shown to have great effectiveness in combination with anti-CD4 mAb [16], modulating T cell activation in collagen induced arthritis in mice. A combined interference with TIRC7 mediated pathways by mAb targeting and TNF-α triggered pathways by soluble TNF-α receptor protein construct revealed a highly beneficial effect on the course of CIA. Both compounds are involved in different signalling pathways and are expressed at different stages in the process of immune activation. Based on the results obtained so far, TIRC7 seems to be predominantly involved in the induction phase of the immune response whereas TNF-α pathway is primarily involved in the effector phase of the immune response corresponding to

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later stages of immune activation. As our data suggest, interference with both pathways results in diminished secretion of IFN-γ as well as TNF-α, both of which are known to promote inflammation, which results in an additive therapeutic effect on arthritis. Moreover, the combined effect on different signalling pathways involved in the inflammatory process may allow the administration of lower doses of each modulatory agent necessary to achieve clinically relevant therapeutic effects, thereby reducing the incidence and the profile of undesired side-effects.

Taken together, these results strongly support the clinical utility of anti-TIRC7 mAb treatment in autoimmune disease, such as RA, making the targeting of TIRC7 a novel approach in the development of new therapies. The effect of anti-TIRC7 mAb on B cell activation might lead to reduction of pathological antibody responses such as immunogenicity to biological compounds which suggests that its efficacy may be broadly generalizeable for this class of disorders. If translated into clinical use, anti-TIRC7 mAb therapy might be beneficial in the treatment of RA and other autoimmune disorders.

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