Immunology and Cell Biology (2012) 00, 1–8

www.nature.com/icb

ORIGINAL ARTICLE

TNFRp55 controls regulatory T cell responses in *Yersinia*-induced reactive arthritis

Ethelina Cargnelutti[1], José L Arias[1], Susana R Valdez[2], Gabriel A Rabinovich[3] and María S Di Genaro[1]

In addition to its well-known pro-inflammatory effects, tumor necrosis factor (TNF) displays anti-inflammatory activities through mechanisms poorly understood. Previously, we reported the development of severe chronic *Yersinia enterocolitica*-induced reactive arthritis (ReA) in mice lacking the TNF receptor (TNFR)p55. As regulatory T (T_{reg}) cells limit chronic inflammation, here we aim to investigate the expansion and function of $CD4^+CD25^+FoxP3^+$ T_{reg} cells in the ReA animal model. The number of T_{reg} cells as well as the *FoxP3* mRNA expression and interleukin (IL)-10 levels were significantly decreased in joint regional lymph nodes (RLNs) of *TNFRp55*$^{-/-}$ mice vs wild-type (WT) mice at the arthritis onset. However, at chronic phase of arthritis, the number of T_{reg} cell in *TNFRp55*$^{-/-}$ was similar to WT mice. To explore the *in vivo* function of T_{reg} cells at this chronic phase in WT and *TNFRp55*-deficient mice, we adoptively transferred $CD4^+$ T cells from *TNFRp55*-deficient mice of day 21, into naïve WT or *TNFRp55*$^{-/-}$ mice. When knockout mice were used as recipients we observed higher delayed-type hypersensitivity (DTH) responses and joint inflammation after heat-killed *Yersinia* (HKY) stimulation. Accordingly, we found higher levels of IL-17, interferon (IFN)-γ, IL-6, transforming growth factor-β1 and IL-12/23p40 and lower IL-10 levels in RLN of paws challenged with HKY in *TNFRp55*$^{-/-}$ recipient mice. In addition, we found that $CD4^+$ T cells from *TNFRp55*$^{-/-}$ mice controlled antigen-specific IL-12/23(p40) production in recipient WT mice. Our results show that TNFRp55 controls the induction and function of T_{reg} cells through differential regulation of cytokine production, suggesting a novel molecular target for immune intervention in ReA.
Immunology and Cell Biology (2012) **0,** 000–000. doi:10.1038/icb.2012.65

Keywords: reactive arthritis; regulatory T cells; TNF; TNFRp55; *Yersinia enterocolitica*

Tumor necrosis factor (TNF) is a pleiotropic cytokine, which has key roles in the initiation and orchestration of inflammation and immunity.[1] However, increasing evidence revealed unexpected immunosuppressive and anti-inflammatory effects for this cytokine. The mechanisms of these contradictory effects remain poorly understood.[2,3] This cytokine binds to the TNF receptor (TNFR) superfamily members TNFR1 (TNFRp55) and TNFR2 (TNFRp75). While TNFRp75 has a restricted expression pattern being present only on hemopoietic and endothelial cells, TNFRp55 is expressed on most cell types and is highly upregulated on antigen-presenting cells (APCs).[4]

Reactive arthritis (ReA) is a particular type of arthritis originated from certain gastrointestinal or genitourinary infections. In previous studies, we reported the development of severe chronic *Yersinia enterocolitica*-induced ReA in mice lacking the TNFRp55.[5] Interestingly, although WT mice showed a peak of acute arthritis on day 14 (arthritis onset), clinical severity was markedly attenuated in comparison with *TNFRp55*$^{-/-}$ mice. In fact, *TNFRp55*$^{-/-}$ mice developed joint inflammation and progressed rapidly toward a chronic arthritogenic process.[5] This study indicated a protective and tempering function of TNFRp55 signaling in the development of *Yersinia*-induced chronic ReA;[5] yet, the mechanisms underlying sustained joint inflammation in *TNFRp55*$^{-/-}$ mice are still uncertain.

$CD4^+CD25^+$ Forkhead box p3 (FoxP3)$^+$ regulatory T (T_{reg}) cells control immune responses to self and foreign antigens in secondary lymphoid organs and at sites of tissue inflammation. T_{reg} cells are largely produced in the thymus (naturally occurring T_{reg} cells; nT_{reg}) and constitute 3–6% of $CD4^+$ T cells in the peripheral compartment.[6] However, FoxP3 can also be induced in $CD4^+$ T cells during ongoing immune responses upon T-cell receptor stimulation or in the presence of transforming growth factor (TGF)-β.[7] Interestingly, TNF selectively activates T_{reg} cells, resulting in proliferation, upregulation of FoxP3 and enhancement of their suppressive activities. In fact, T_{reg} cells as opposed to $CD25^-$ conventional T cells constitutively express high levels of TNFRp75, but do not express TNFRp55.[8] Thus, through binding to TNFRp75, TNF may enhance T_{reg} cell activity and may help limit collateral damage caused by exuberant immune responses.[8] We hypothesized that TNFRp55 signaling may control T_{reg}

[1]Division of Immunology, Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis; Laboratory of Immunopathology, Multidisciplinary Institute of Biological Investigations—San Luis (IMIBIO-SL), National Council of Scientific and Technical Investigations (CONICET), San Luis, Argentina; [2]Laboratory of Reproduction and Lactation, CONICET; Science Basics Institute of National University of Cuyo, Mendoza, Argentina and [3]Laboratory of Immunopathology, Institute of Biology and Experimental Medicine (IBYME), CONICET; Faculty of Exact and Natural Sciences, University of Buenos Aires, Buenos Aires, Argentina
Correspondence: Dr MS Di Genaro, Laboratorio de Immunopatología, Universidad Nacional de San Luis, Ejército de los Andes 950, San Luis 5700, Argentina.
E-mail: sdigena@unsl.edu.ar
Received 26 February 2012; revised 29 October 2012; accepted 29 October 2012

cell expansion and suppressive activity as *TNFRp55*$^{-/-}$ mice developed chronic arthritis even in the presence of TNFRp75.

The present work was conducted to investigate the T_{reg} cell compartment in mesenteric lymph nodes (MLNs) and joint regional lymph nodes (RLNs) of *Yersinia*-infected *TNFRp55*$^{-/-}$ and WT mice as a potential underlying mechanism of chronic arthritis in this animal model. The results presented here may contribute to identify a novel regulatory mechanism mediated by the TNF-TNFRp55 axis. Additionally, our findings may have important implications in pathologic settings as the ReA model recapitulates several clinical features experienced by patients exposed to continuous TNF blockade therapy.

RESULTS

TNFRp55 signaling regulates T_{reg} cell frequency and *FoxP3* mRNA expression in RLNs of ReA mice

To examine whether TNFRp55 signaling controls T_{reg} cell frequency in local inflammatory microenvironments, we determined the frequency of T_{reg} cells in MLNs and RLNs of *TNFRp55*$^{-/-}$ and WT mice, after 7, 14 and 21 days of *Yersinia* infection. The lymphoid cell population was obtained from MLNs or RLNs from both groups of mice, stained with fluorescently conjugated antibodies against CD4, CD25 and FoxP3 and analyzed by flow cytometry. The percentages of Treg cells observed in MLN and RLN from WT mice were in line with those observed by previous studies.[9,10] We could find no significant differences in the frequency or absolute number of T_{reg} cells in MLNs from *TNFRp55*$^{-/-}$ and WT mice collected at different times post-infection (Figures 1a and d). However, a significantly lower frequency of T_{reg} cells was detected in RLNs from *TNFRp55*$^{-/-}$ mice 14 days after *Yersinia* infection ($3.6 \pm 0.1\%$ and $5.1 \pm 0.4\%$ in *TNFRp55*$^{-/-}$ and WT mice, respectively) ($P<0.05$ vs WT mice; Figures 1b and c). This lower frequency correlated with a reduced absolute number of T_{reg} cells in RLNs of *TNFRp55*$^{-/-}$ mice (Figure 1e). In contrast, *TNFRp55*$^{-/-}$ mice showed a significantly higher frequency of T_{reg} cells in RLNs, compared with WT mice 21 days after infection ($4.4 \pm 0.3\%$ and $3.4 \pm 0.3\%$, respectively) ($P<0.05$; Figures 1b and c). However, equivalent numbers of T_{reg} cells were observed in *TNFRp55*$^{-/-}$ and WT mice at day 21 after infection (Figure 1e). This similar number might reflect a differential expansion of T_{eff} cells in RLN of *TNFRp55*$^{-/-}$ and WT mice.

As FoxP3 is a distinctive transcription factor mostly expressed by $CD4^+$ $CD25^+$ T_{reg} cells, we then studied whether the lower T_{reg} cell frequency observed in RLN from *TNFRp55*$^{-/-}$ mice 14 days after *Yersinia* infection correlated with changes in *FoxP3* mRNA expression. Fourteen days after infection, $CD4^+$ T cells from *TNFRp55*$^{-/-}$ and WT mice were purified from RLNs and used for RT-qPCR analysis. *FoxP3* mRNA expression of *TNFRp55*$^{-/-}$ mice was significantly lower compared with their WT counterpart (relative expression 0.78 ± 0.03 vs 1.18 ± 0.03, respectively) ($P<0.005$; Figure 2). Thus, TNFRp55 signaling controls the number of $CD4^+CD25^+$ T_{reg} cells in a time-dependent manner in local inflammatory microenvironments.

TNFRp55 signaling controls interleukin-10 production in mucosal and RLNs of *Yersinia*-infected mice

Since interleukin (IL)-10 is a critical immunosuppressive cytokine produced by T_{reg} cells, we then investigated whether the absence of TNFRp55 signaling influences IL-10 production in both MLNs and RLNs after 7, 14 and 21 days of *Yersinia* infection. Notably, lack of TNFRp55 led to substantial differences in the kinetics of IL-10 (Figure 3). IL-10 peaked earlier in MLNs of *TNFRp55*$^{-/-}$ mice, as shown by its expression levels on day 7 after *Yersinia* infection ($P<0.005$ compared with MLNs of WT mice; Figure 3a). In contrast, IL-10 augmented in MLNs of WT mice only 21 days after infection ($P<0.05$ vs MLNs of *TNFRp55*$^{-/-}$ mice; Figure 3a). Conversely, in RLNs, the IL-10 peak was observed on day 14 post infection in WT mice ($P<0.05$ vs RLNs of *TNFRp55*$^{-/-}$ mice; Figure 3b). Interestingly, this time point correlates with the onset of the arthritogenic process.[5] However, higher amounts of IL-10 were detected on days 7 and 21 post infection in RLNs from *TNFRp55*$^{-/-}$ mice ($P<0.05$ and $P<0.005$ vs WT mice; Figure 3b). These results indicate time-dependent modulation of IL-10 production mediated by TNFRp55 signaling.

TNFRp55 tunes the suppressive function of T_{reg} cells *in vivo*

To investigate whether TNFRp55 deficiency affects the suppressive function of T_{reg} cells *in vivo*, we performed adoptive transfer experiments of $CD4^+$ T cells into WT and *TNFRp55*$^{-/-}$ mice. Since WT mice successfully controlled chronic ReA, we used only TNFRp55 knockout mice as cell donors. However, we used knockout and WT as recipient mice, to study whether T_{reg} cells from *TNFRp55*$^{-/-}$ mice could be influenced by the TNFRp55-deficient microenvironment. We obtained $CD4^+$ T cells from spleen or RLNs of *TNFRp55*$^{-/-}$ mice, 21 days after *Yersinia* infection (chronic phase of arthritis). $CD4^+$ T cells were adoptively transferred into syngeneic *TNFRp55*$^{-/-}$ or WT mice ($n=5$ mice/group) by intraperitoneal injection. After cell transfer, *in vivo* T-cell responses were examined in both groups of mice by delayed-type hypersensitivity (DTH) reactions induced by intraplantar injection of heat-killed *Yersinia* (HKY) in one paw or phosphate buffer saline (PBS) in the other. After 24 h, DTH response was evaluated, and RLN and ankle joints were removed for cytokine measurement and histopathological assessment, respectively. Controls included *TNFRp55*$^{-/-}$ or WT mice that were not transferred with $CD4^+$ T cells. *TNFRp55*$^{-/-}$ recipient mice showed significantly higher DTH responses following transfer of $CD4^+$ T cells as compared with those observed in WT recipient mice (0.55 ± 0.08 mm and 0.03 ± 0.03 mm, respectively) ($P<0.05$). Moreover, *TNFRp55*$^{-/-}$ recipient mice showed significantly higher DTH compared with non-transferred *TNFRp55*$^{-/-}$ or WT mice (0.18 ± 0.10 and 0.02 ± 0.02, respectively) ($P<0.05$ vs *TNFRp55*$^{-/-}$ control and $P<0.005$ vs WT control; Figure 4a).

Next, we tested whether transfer of $CD4^+$ T cells induced considerable histopathological changes after HKY or PBS injection. Joints from *TNFRp55*$^{-/-}$ recipient mice corresponding to flanks receiving HKY challenge showed substantially higher total histological score index, compared with the respective joints of WT recipient mice (3.9 ± 0.31 and 2.4 ± 0.46, respectively) ($P<0.05$; Figure 4b). Both *TNFRp55*$^{-/-}$ and WT recipient mice showed HKY-specific joint histological changes since total score index was not significant in PBS flanks (1.1 ± 0.38 and 0.33 ± 0.17 in *TNFRp55*$^{-/-}$ and WT recipient mice, respectively) and significantly differences were detected when compared in HKY vs PBS flanks in *TNFRp55*$^{-/-}$ and WT mice ($P<0.001$ and $P<0.05$, respectively; Figure 4b).

Representative histopathological changes observed in each joint of both groups of mice are shown in Figure 4c. Thus, in contrast to flanks injected with PBS, joints from WT mice corresponding to flanks challenged with HKY, showed slight disorganization of the synovial membrane (Figure 4c). Similar slight alterations of the synovial membrane and cartilage were observed after histopathological evaluation of joints from *TNFRp55*$^{-/-}$ recipient mice corresponding to flanks receiving PBS alone (Figure 4c). Moreover, disorganization of the synovial membrane, proliferation of synovial lining cells with pannus formation and bone/cartilage degradation

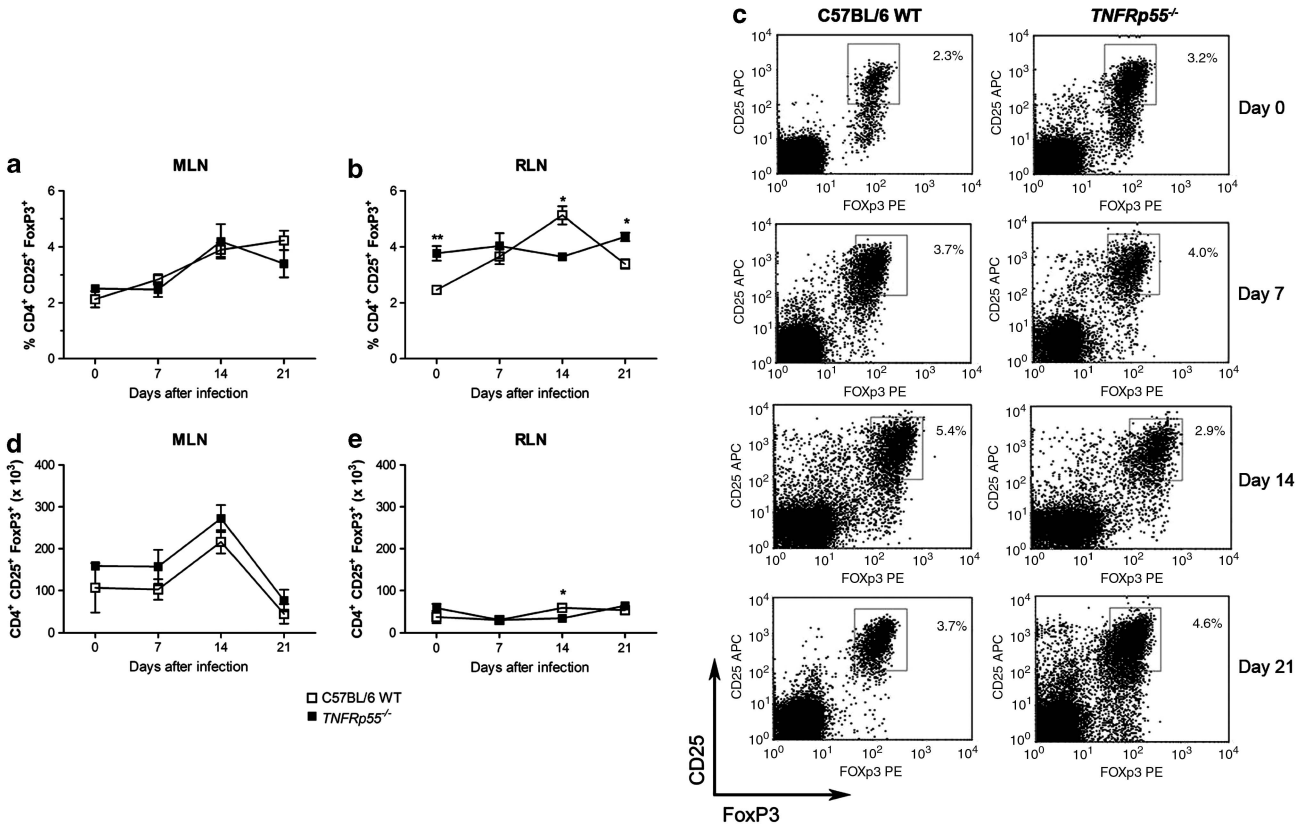

Figure 1 Frequency and absolute number of CD4$^+$CD25$^+$FoxP3$^+$ T_{reg} cells in lymph nodes after *Y. enterocolitica* infection. Six- to eight-week-old C57BL/6 WT or *TNFRp55*$^{-/-}$ mice were orally infected with *Y. enterocolitica* O:3. Before and at 7, 14 and 21 days after infection, T_{reg} cells were analyzed by flow cytometry from single cell suspension of MLNs (**a**) and joint RLNs (**b**). The density plots show the frequency of CD25$^+$ FoxP3$^+$ cells from a CD4$^+$ gate of RLN from a representative mouse of each group (**c**). (**d**, **e**) The absolute number of T_{reg} cells in MLN and RLR, respectively, is shown. In (**a**), (**b**), (**d**) and (**e**), data are reported as mean ± s.e.m for each day. Data are representative of two independent experiments with 3–5 mice per group. *Indicates statistically significant differences ($P<0.05$).

were clearly evident when joints from *TNFRp55*$^{-/-}$ recipient mice corresponding to flanks challenged with HKY were histopathologically assessed (Figure 4c).

Adoptive transfer of CD4$^+$ T cells from *TNFRp55*$^{-/-}$ mice alters cytokine responses in recipient mice

To evaluate cytokine responses induced by adoptive transfer of CD4$^+$ T cells from *TNFRp55*$^{-/-}$ mice, we obtained homogenates from RLN corresponding to flanks from *TNFRp55*$^{-/-}$ or WT recipient mice receiving intraplantar injection of PBS or HKY. The amounts of IL-17, interferon (IFN)-γ, IL-6, TGF-β1, IL-12/23p40 subunit and IL-10 were determined by capture ELISA. RLN from *TNFRp55*$^{-/-}$ recipient mice corresponding to flanks challenged with HKY showed significantly higher IL-17 production compared with RLN from flanks of WT recipient mice receiving PBS ($P<0.05$; Figure 5a).

RLNs from *TNFRp55*$^{-/-}$ recipient mice obtained from flanks challenged with HKY showed significantly higher amounts of IFN-γ and IL-6, compared with *TNFRp55*$^{-/-}$control, WT control and WT recipient mice receiving PBS ($P<0.05$ vs *TNFRp55*$^{-/-}$control vs WT control and vs WT recipient mice; Figures 5b and c). Notably, RLN from *TNFRp55*$^{-/-}$ recipient mice corresponding to flanks challenged with HKY showed significantly higher levels of TGF-β1, compared with *TNFRp55*$^{-/-}$ and WT control receiving PBS or HKY, respectively ($P<0.05$ vs *TNFRp55*$^{-/-}$ control and WT control mice, Figure 5d). Significantly lower amounts of TGF-β1 were found in

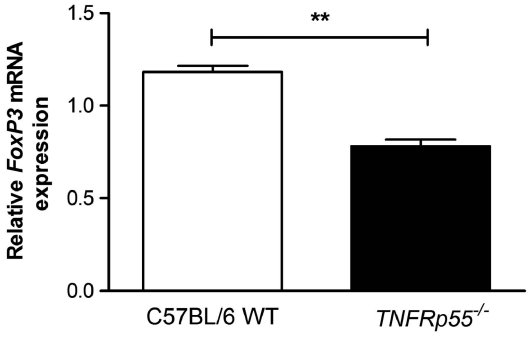

Figure 2 *FoxP3* mRNA expression in CD4$^+$ T cells from RLN. C57BL/6 WT or *TNFRp55*$^{-/-}$ mice were orally infected with *Y. enterocolitica* O:3. Fourteen days after infection (arthritis onset), *FoxP3* mRNA expression was analyzed by RT-qPCR from CD4$^+$ cells of RLN. CD4$^+$ cells were positive enriched by magnetic labeling. Uninfected age-matched mice were used as control. The shown RT-qPCR data were normalized to mouse β-*actin* gene. Data represent two independent experiments with five mice per group. **Indicates statistically significant differences ($P<0.005$).

TNFRp55$^{-/-}$ control mice receiving PBS compared with RLN from WT recipient mice challenged with HKY ($P<0.05$, Figure D). However, no significant differences in IL-17, IFN-γ, IL-6 and TGF-β1 were found between WT mice (control and recipient mice),

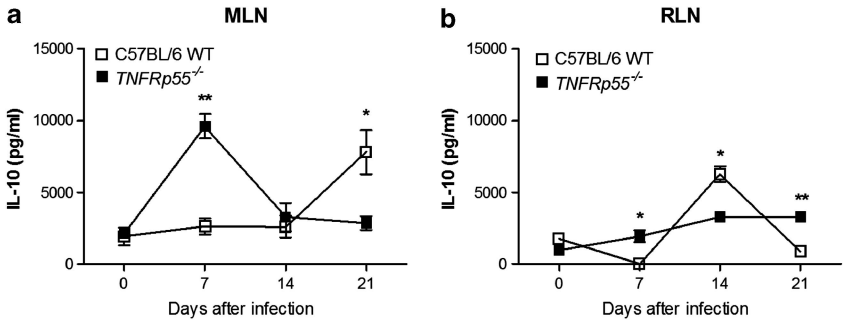

Figure 3 IL-10 production in C57BL/6 WT and *TNFRp55*$^{-/-}$ mice after *Y. enterocolitica* infection. C57BL/6 WT and *TNFRp55*$^{-/-}$ mice were orally infected with *Y. enterocolitica* O:3. Before and at days 7, 14 and 21 after infection, IL-10 production was measured in MLNs (**a**) and in joint RLNs (**b**) by ELISA. Data represent the media ± s.e.m of three independent experiments. Five mice per group and day were used (6–8 weeks old). *Indicates statistically significant differences (*$P<0.05$, **$P<0.005$).

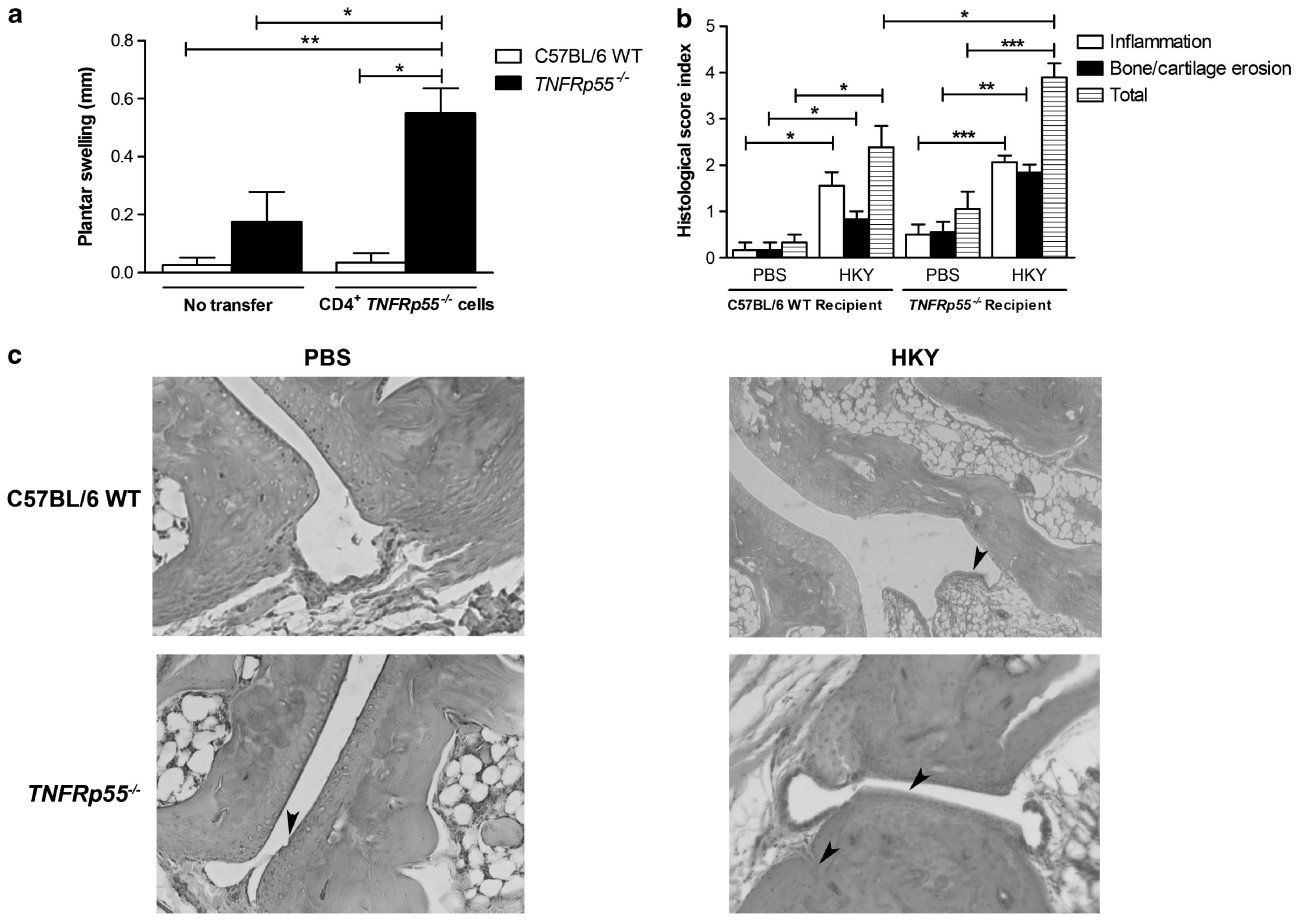

Figure 4 Analysis of adoptive transfer of CD4$^+$ T cells from *TNFRp55*$^{-/-}$ mice. *TNFRp55*$^{-/-}$ mice were infected with *Y. enterocolitica* O:3. On day 21 after infection, CD4$^+$ T cells were enriched by magnetic labeling and then transferred into naïve C57BL/6 (white) or *TNFRp55*$^{-/-}$ (black) mice and next challenged with HKY intraperitoneally. (**a**) *In vivo* DTH responses in plantar paws of naïve C57BL/6 WT or *TNFRp55*$^{-/-}$ recipient mice. DTH responses against HKY were measured as the difference between HKY and PBS control paws and data are expressed as mean ± s.e.m. Control mice of each group did not receive CD4$^+$ T cells (no transfer). (**b**) Histological score index of C57BL/6 WT and *TNFRp55*$^{-/-}$ recipient mice, after adoptive transfer of CD4$^+$ T cells from *TNFRp55*$^{-/-}$mice. (**c**) Representative sections of joints of C57BL/6 WT and *TNFRp55*$^{-/-}$ mice transferred with CD4$^+$ cells from *TNFRp55*$^{-/-}$ mice and injected with PBS or HKY (H&E, original magnification ×10). *Indicates statistically significant differences (*$P<0.05$, **$P<0.005$, ***$P<0.001$). Arrowheads indicate slight disorganization of the synovial membrane in HKY-treated WT mice, cartilage erosion in both PBS and HKY-treated paws from *TNFRp55*$^{-/-}$, and pannus in HKY-treated paw from *TNFRp55*$^{-/-}$ mice.

whether homogenates were obtained from RLN from flanks receiving PBS or HKY (Figures 5a–d).

RLN from WT recipient mice corresponding to flanks receiving HKY showed significantly higher levels of IL-10, compared with *TNFRp55*$^{-/-}$ recipient receiving PBS or *TNFRp55*$^{-/-}$ control mice challenged with HKY ($P<0.005$ vs *TNFRp55*$^{-/-}$ recipient mice and $P<0.05$ vs *TNFRp55*$^{-/-}$ control mice; Figure 6a). WT recipient mice challenged with HKY also showed significantly higher amounts of

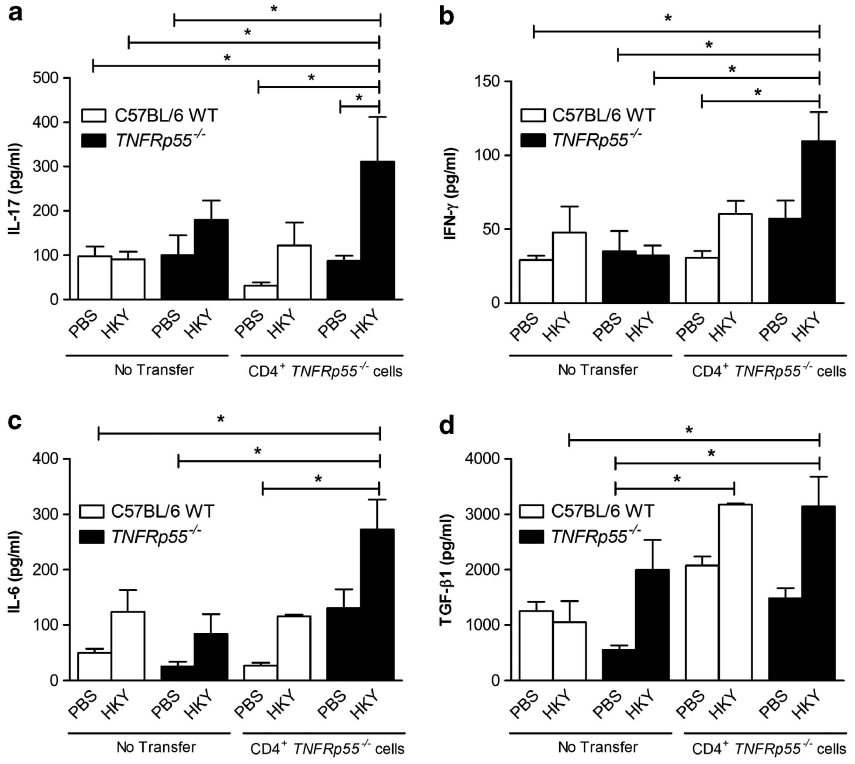

Figure 5 Cytokine production by RLN of arthritic joints following adoptive transfer of *TNFRp55*$^{-/-}$ CD4$^+$ T cells. Naïve C57BL/6 WT and *TNFRp55*$^{-/-}$ mice were adoptively transferred with of CD4$^+$ T cells from *TNFRp55*$^{-/-}$ mice; 48 h after HKY-intraplantar challenge, RLN was removed and IL-17 (**a**), IFN-γ (**b**), IL-6 (**c**) and TGF-β1 (**d**) were measured by ELISA. Mice which did not received CD4$^+$ T cells (non-transferred) were used as controls. Data are representative of two independent experiments with five mice per group (6–8 weeks old). *Indicates statistically significant differences (*$P<0.05$).

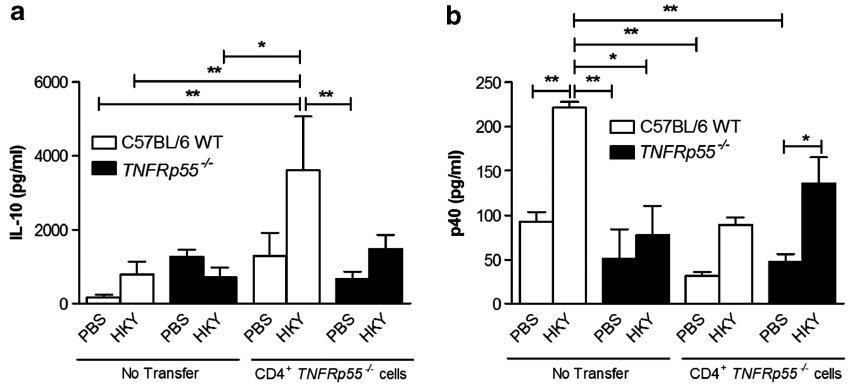

Figure 6 IL-10 and ILp12/23p40 in joint RLN following adoptive transfer of CD4$^+$ T cells from *TNFRp55*$^{-/-}$mice. Naïve C57BL/6 WT and *TNFRp55*$^{-/-}$ mice were adoptively transferred with CD4$^+$ T cells from *TNFRp55*$^{-/-}$ mice; 48 h after HKY-intraplantar challenge, RLN was removed and IL-10 (**a**) and IL-12/23p40 (**b**) were measured by ELISA. Mice which did not received CD4$^+$ T cells (non-transferred) were used as controls. Data are representative of two independent experiments with five mice per group (6–8 weeks old). *Indicates statistically significant differences (*$P<0.05$, **$P<0.005$).

IL-10 compared with WT control mice, whether they were challenged with HKY or PBS ($P<0.05$ vs WT PBS control and vs WT HKY control; Figure 6a).

Finally, *TNFRp55*$^{-/-}$ recipient mice challenged with HKY showed higher amounts of IL-12/IL-23p40 subunit compared with *TNFRp55*$^{-/-}$ recipient mice given PBS ($P<0.05$; Figure 6b). Interestingly, control WT mice receiving HKY showed higher amounts of IL-12/IL-23p40 compared with control WT mice receiving PBS, *TNFRp55*$^{-/-}$ control mice receiving PBS or HKY, and in both WT and *TNFRp55*$^{-/-}$ recipient mice receiving PBS ($P<0.005$ vs PBS WT control, $P<0.005$ vs PBS *TNFRp55*$^{-/-}$ control mice, $P<0.05$ vs HKY *TNFRp55*$^{-/-}$ control mice and $P<0.005$ vs PBS WT and *TNFRp55*$^{-/-}$ recipient mice; Figure 6b).

DISCUSSION

The cytokine TNF exerts pro-inflammatory effects in many autoimmune diseases including rheumatoid arthritis, Crohn's disease, ulcerative colitis and psoriasis. Nevertheless, mounting evidence indicates that TNF also has substantial anti-inflammatory effects.[11] In this regard, injection of TNF decreased the severity of lupus,[12] diabetes,[13] adjuvant-induced arthritis[3] and experimental autoimmune

encephalomyelitis[14] in different experimental models. In humans, TNF antagonists are effective in a large number of patients with rheumatoid arthritis. However, TNF antagonist therapy resulted in induction of lupus-like syndromes in few rheumatoid arthritis cases,[11] and it has been shown to exacerbate disease in some patients with psoriasis.[11] The mechanisms underlying the dual pro-inflammatory and anti-inflammatory effects of TNF, as well as the occasionally paradoxical effects of TNF antagonists in autoimmune diseases remain incompletely understood.[15]

Recently, we reported that mice lacking TNFRp55 develop severe chronic ReA after oral infection with *Yersinia*.[5] As T_{reg} cells have crucial roles in limiting chronic inflammation and TNF has been shown to promote T_{reg} cell expansion through binding to TNFRp75,[15] we investigated the frequency of T_{reg} cells in *TNFRp55*$^{-/-}$ mice with *Yersinia*-induced ReA to evaluate the association between chronic arthritis with dysregulation of immune responses. We demonstrate that TNFRp55 deficiency influences T_{reg} cell frequency and regulatory capacity in ReA, suggesting a suitable model for investigating the impact of TNFRp55 deficiency in T_{reg} cell function in a pro-inflammatory environment.

Several studies reported the presence of T_{reg} cells induced upon chronic antigen exposure in gut-associated lymphoid tissues including MLN.[16] Since in our study ReA developed after oral *Yersinia* infection, and bacterial antigen persistence in the mucosa has been suggested for chronic ReA,[17] we explored the frequency of T_{reg} cells in MLN; however, we could find no differences in T_{reg} cell induction between *TNFRp55*$^{-/-}$ and WT mice, suggesting that intestinal T_{reg} cell induction is TNFRp55-independent at least in our animal model. In contrast, our results in RLN showed that on day 14, which corresponds to arthritis onset in *TNFRp55*$^{-/-}$ mice and to acute joint inflammation in WT mice,[5] local T_{reg} cell frequency and absolute number was lower in *TNFRp55*$^{-/-}$ mice. In addition, we found decreased *FoxP3* mRNA expression in CD4$^+$ T cells infiltrating RLN that correlated with suppressive activity of T_{reg} cells.[18] T_{reg} cell deficiency during the arthritis onset influenced considerably the arthritogenic process as severe arthritis was observed in *TNFRp55*$^{-/-}$ mice at day 21 after infection,[5] even when a similar number of T_{reg} cells in RLN were found at that time. This finding may be due to the increased resistance of effector T cells (Th1 or Th17 cells) to T_{reg} cell-induced immunosuppression. Likewise, in a murine model of type 1 diabetes, pancreatic islet-specific effector T cells (T_{eff}) boosted the expansion of T_{reg} cells in a TNF-dependent manner.[19] In addition, a very recent study showed that naturally occurring T_{reg} (nTreg) cells require TNF through TNFRp75 for its optimal function[20] Hence, as the arthritogenic process is more severe in *TNFRp55*$^{-/-}$ mice in the ReA animal model,[5] and the T_{reg} cell number is altered in these mutant mice, we concluded that the TNFRp55 deficiency considerably influences the T_{reg} cell compartment. In this regard, Murai *et al.*[18] have demonstrated that IL-10 acts directly on T_{reg} cells to maintain *FoxP3* expression, which is otherwise lost under inflammatory conditions. Our data show a strong correlation between altered IL-10 production, T_{reg} cell frequency and *FoxP3* expression in *TNFRp55*$^{-/-}$ mice, indicating a central role of TNFRp55 signaling in inducing IL-10 and consequently maintaining T_{reg} cells for controlling *Yersinia*-induced ReA. Future studies are being conducted to elucidate the link between altered T_{reg} cell frequency and IL-10 production in TNFRp55-deficient mice. On the other hand, we have recently demonstrated that TNFRp55 signaling modulates macrophage functions in response to *Yersinia* lipopolyssacharide stimulation.[21] Since T_{eff} and T_{reg} cell subsets do not express TNFRp55,[8] this signaling effect may negatively regulate the inflammatory response of non-lymphoid cells through differential modulation of anti-inflammatory or pro-inflammatory cytokines.

Our results indicate that *Yersinia*-induced ReA in *TNFRp55*$^{-/-}$ mice is mediated by CD4$^+$ T cells as *TNFRp55*$^{-/-}$ recipient mice transferred with these cells developed higher *Yersinia*-specific DTH response and joint inflammation. Similarly, antigen-induced arthritis,[22] Lyme arthritis in *TLR2*$^{-/-}$ mice[23] and arthritis in SKG mice[24] are all mediated by CD4$^+$ T cells. In the present study, we confirmed exacerbated arthritis in *TNFRp55*$^{-/-}$ but not in WT naïve mice transferred with CD4$^+$ T cells from *TNFRp55*-deficient mice after HKY challenge suggesting *in vivo* dysregulated T cell response even when T_{reg} cells were present in the pool of CD4$^+$ transferred cells. Importantly, in addition to TNFRp55 deficiency antigen stimulation appears to be necessary for the development of severe arthritis.

A successful immune defense strategy requires intricate negative regulation to restrict host tissue damage caused by over-exuberant inflammation.[25] In this regard, T_{reg} cell deficiency causes an inflammatory syndrome characterized by massive cytokine storm including increased amounts of Th1, Th2 and Th17 cytokines.[25] Previously, we demonstrated that in the absence of TNFRp55 signaling, local Th1 and Th17 effectors cells are dysregulated.[5] In this work, we confirmed these findings in RLN from *TNFRp55*$^{-/-}$ and WT mice transferred with CD4$^+$ T cells. In accordance, we found increased amounts of Th17-related cytokines including IL-17, IL-6 and TGF-β1 and the Th1-related cytokine IFN-γ when *TNFRp55*$^{-/-}$ mice were used as recipients, suggesting that TNFRp55 deficiency influences the ability of T_{reg} cells to control Th1 and Th17 responses. In this regard, T_{reg} cells suppress Th1 and Th17 cell-mediated inflammation.[26,27] Moreover, adoptive transfer experiments showed decreased amounts of IL-10 in *TNFRp55*$^{-/-}$ as compared with WT recipient mice supporting a central role of TNFRp55 signaling on IL-10 induction, which is critical for suppression of Th17 cell-mediated inflammation.[25,28]

A common denominator to divergent Th1 and Th17 effectors subsets is the p40 common chain that is shared by the Th1-promoting cytokine IL-12 and by the Th17-promoting cytokine IL-23. Hence, we examined the expression of p40 in RLN of *TNFRp55*$^{-/-}$ and WT recipient mice and found that, in contrast to WT mice, *TNFRp55*$^{-/-}$ mice could not control p40 expression after HKY challenge. This result indicates a role of TNFRp55 signaling in the regulation of IL-12 and IL-23 synthesis as previously suggested.[29–31] Unexpectedly, we found increased amounts of p40 in RLN of non-transferred WT mice after HKY challenge, which was in line with the decreased IL-10 concentration. Our results indicate that the source of IL-10 are T_{reg} cells present in transferred CD4$^+$ T cells, which display suppressive function on p40 secretion when TNFRp55 is present in recipient mice. Thus, a TNF-mediated mechanism may limit pro-inflammatory Th1 and Th17 responses and may indirectly control T_{reg} cell frequency and function.

Does TNFRp75 have any regulatory role in *TNFRp55*$^{-/-}$ mice? Although further studies are needed to fully address this question, it has been demonstrated that T_{eff}[8,32] and T_{reg}[8,33] cells express high levels of cell surface TNFRp75. Moreover, TNF has a stimulatory effect on T_{reg} cell expansion and activity through binding to TNFRp75.[8,34] Our findings based on adoptive transfer of CD4$^+$ T cells from TNFRp55-deficient mice into WT or *TNFRp55*$^{-/-}$ naïve mice suggest that these cells are resistant to immune regulation in *TNFRp55*$^{-/-}$ recipient mice, suggesting that TNFRp75 signaling on T_{reg} cells was not effective in controlling T_{eff} cells in the absence of TNFRp55. However, we cannot exclude the possibility that TNFRp75 might enhance the capacity of T_{eff} cells to resist T_{reg}-mediated

suppression.[35] This issue warrants further studies in ReA and other chronic inflammation models. On the other hand, it has been reported that unlike TNFRp75, surface expression of TNFRp55 is barely detectable on either resting or activated T_{eff} or T_{reg} cells.[8] Thus, decreased T_{reg} cell activity observed in *TNFRp55*$^{-/-}$ mice at day 14 after *Y. enterocolitica* infection cannot be attributed to impaired signaling via TNFRp55 on T cells. In contrast, the immunoregulatory actions mediated by TNFRp55 appear to be limited to the APC compartment.[30]

In conclusion, our findings demonstrate that TNFRp55 signaling modulates the anti-inflammatory and pro-inflammatory cytokine balance and influences T_{reg} cell differentiation and activity to control chronic ReA. Our findings also offer a note of warning to patients receiving anti-cytokine therapies, particularly in ReA, as they may lead to unpredicted effects including skewing the immune response toward unexpected pro-inflammatory responses and negative regulation of T_{reg} cell function.

METHODS

Mice

TNFRp55-deficient mice (*TNFRp55*$^{-/-}$) on a C57BL/6 background were kindly provided by Max von Pettenkofer Institute (Munich, Germany). C57BL/6 wild-type (WT) mice were purchased from the Animal Facilities of National University of La Plata (La Plata, Argentina). Breeding colonies were established at the Animal Facility of the National University of San Luis (San Luis, Argentina). Mice were kept under specific pathogen-free conditions in a positive-pressure cabinet (EHRET, Emmendingen, Germany) and provided with sterile food and water *ad libitum*. Six- to eight-week-old mice were used for the experiments. Three independent experiments were carried out with 3–9 mice per group. All animal procedures were performed according to the rules and standards for the use of laboratory animals of the National Institute of Health, USA. Animal experiments were approved by the Institutional Committee of Care and Use of Animals (CICUA) of Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis (San Luis, Argentina).

Bacterial culture and infection

Strain MCH 700 of *Y. enterocolitica* O:3 (kindly provided by Dr Kapperud, Department of Bacteriology, Oslo, Norway) was used for infection. Bacteria were cultured as described earlier.[36] Mice were starved for 8 h, and were then infected orogastrically with $1–5 \times 10^8$ *Yersiniae* in 200 µl of sterile PBS, using a gastric tube. The number of inoculated bacteria was controlled by plating serial dilutions of the inoculated suspension on Mueller–Hinton agar and counting the colony-forming units after incubation at 26 °C for 48 h.

Cytokine determination by ELISA

At days 7, 14 and 21 after *Yersinia* infection, mouse IL-10 was determined in MLN and RLN (inguinal and popliteals lymph nodes) homogenates and prepared as described previously.[5] After adoptive transfer of CD4$^+$ *TNFRp55*$^{-/-}$ cells and DTH response, IL-10, IL-17, IFN-γ, IL-6, TGF-β1 and IL-12/23p40 were determined in RLN homogenates from recipient mice. Cytokines were determined using capture ELISA kits (eBioscience, San Diego, CA, USA) according to manufacturer's instructions. The sensitivity of the assays was 30 pg ml^{-1} for IL-10, 4 pg ml^{-1} for IL-17, 15 pg ml^{-1} for IFN-γ, 4 pg ml^{-1} for IL-6, 60 pg ml^{-1} for TGF-β1 and 2 pg ml^{-1} for IL-12/23 p40.

Flow cytometry

At days 7, 14 and 21 after *Yersinia* infection, MLN and RLN were mechanically disrupted and filtered through a 70-µm cell strainer (BD FALCON cell Strainer). Cells were stained using a Mouse Regulatory T Cell Staining Kit (eBioscience) according to manufacturer's instructions. Kit includes the following conjugated antibodies: FITC anti-mouse CD4 (RM4-5), APC anti-mouse CD25 (PC61.5), PE anti-mouse FoxP3 (FJK-16s), anti-mouse CD16/32 (Fc Block) and PE Rat IgG2a isotype control. Data were acquired on a FACScalibur cytometer and analyzed using BD Cell Quest Pro Software (BD Biosciences, San Jose, CA, USA).

Real-time PCR analysis for FoxP3

After 14 days of *Yersinia* infection, CD4$^+$ T cells were purified from RLN via positive selection, using BD IMag anti-mouse CD4 particles according to manufacturer's protocol (BD Biosciences). RLN CD4$^+$ T cells from uninfected mice were used as calibrator. RLN purified CD4$^+$ T cells were then placed immediately in 1 ml of TRIZOL reagent (Invitrogen) and total RNA extraction was performed as described by the manufacturer. Two micrograms of total RNA was reverse-transcribed to obtain complimentary DNA using oligo(dT)$_{15}$ as primers and MMLV retrotranscriptase.[37] Primers were designed to span an intron of the genomic sequence. Mouse β-*actin* forward primer sequence: AGAGGGAAATCGTGCGTGAC; mouse β-*actin* reverse primer sequence: CAATAGTGATGACCTGGCCGT. Mouse *FoxP3* forward primer sequence: TTTACTCGCATGTTCGCCTACTT; Mouse *FoxP3* reverse primer sequence: CTCAAATTCATCTACGGTCCACACT. Real-time reverse transcription PCR (RT-PCR) was carried out using an ABI Prism 7000 apparatus (Applied Biosystems, Foster City CA, USA) and SYBER Green Master Mix solution (Applied Biosystems). PCR condition began with a 10-min 95 °C enzyme activation step, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Expression for *FoxP3* was normalized to β-*actin* expression. Relative gene expression was calculated using $2^{-\Delta\Delta CT}$ method.

Adoptive transfer experiments and DTH response

CD4$^+$ T cells were isolated from the spleen and RLN of *TNFRp55*$^{-/-}$ mice after 21 days of *Yersinia* infection by magnetic beads using BD IMag anti-mouse CD4 particles according to manufacturer's instructions (BD Biosciences). The purity of CD4$^+$ T cells (~95%) was evaluated by flow cytometry. C57BL/6 WT or *TNFRp55*$^{-/-}$ recipient mice received 2×10^6 CD4$^+$ T cells from *TNFRp55*$^{-/-}$ mice by intraperitoneal injection in 100 µl of PBS. This strategy is based on the fact that CD4$^+$ T cells do not express TNFRp55, thereby allowing the possibility to examine the lack of TNFRp55 in the APC inflammatory microenvironment of WT or *TNFRp55*$^{-/-}$ recipient hosts. Two days later, recipient mice were challenged by intraperitoneal injection with HKY. DTH response was carried out 2 days after challenge, with a plantar injection of HKY in the right paw as antigen-specific swelling, and with PBS in the left one as control. DTH was measured with calipers as plantar swelling by substracting the size of the PBS-injected paw from the size of HKY-injected paw. Twenty-four hours after DTH response evaluation, RLN and joints were removed from recipient mice and processed for cytokine determination by ELISA and histopathological examination, respectively. Mice which were not given CD4$^+$ T cells (non-transferred) were used as controls.

Histopathological evaluation

Histopathological examination of the joints was carried out in mice receiving CD4$^+$ T cells from *TNFRp55*$^{-/-}$ mice after DTH evaluation. After routine fixation, decalcification, and paraffin embedding, 5-µm-thick sections were cut and stained with H&E. Histopathological scoring was performed as described by Kyo *et al*.[38] Ankles from different groups of mice were assigned inflammation scores of 0–5: 0, normal; 1 minimal infiltration; 2, mild infiltration; 3, moderate infiltration; 4, marked infiltration; and 5, severe infiltration. The same ankles were assigned a score for cartilage/bone resorption according to the following criteria: 0, normal; 1, minimal (small areas of resorption); 2, mild (numerous areas of resorption); 3, moderate (obvious resorption of trabecular and cortical bone without full-thickness defects in cortex; loss of some trabecule); 4, marked (full-thickness defects in cortical bone and marked trabecular bone loss, without distortion of the remaining cortical surface profile); and 5, severe (full-thickness defects in cortical bone and marked trabecular bone loss, with distortion of the remaining cortical surface profile). The total score was defined as the sum of the scores of inflammation taking into account cartilage and bone destruction. Each slide was score by two independent observers and the average score was recorded.

Statistical analysis

Data are expressed as mean ± s.e.m. Experimental groups were tested for statistically significant differences with the one-way ANOVA following Tukey's Multiple Comparison Test and two-tailed, paired Student's *t*-test, as appropriate. A *P*-value of <0.05 was considered as statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Agency for Promotion of Science and Technology (PICT 2006-838; 2008-763; 2010-870), the National University of San Luis (Project 0401), the Florencio Fiorini Foundation, and the Sales Foundation. SRV, GAR and MSDG are members of the Scientific Career of the National Council of Scientific and Technical Investigations (CONICET); EC and JLA are fellows from the CONICET. We thank Vicente Ragusa for providing wild-type mice for cellular transfer experiments.

1 Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 2003; **3**: 745–756.
2 Chen X, Oppenheim JJ. TNF-α: an activator of CD4$^+$FoxP3$^+$TNFR2$^+$ regulatory T cells. *Curr Dir Autoimmun* 2010; **11**: 119–134.
3 Kim EY, Chi HH, Rajaiah R, Moudgil KD. Exogenous tumor necrosis factor-α induces suppression of autoimmune arthritis. *Arthritis Res Ther* 2008; **10**: R38.
4 Peschon JJ, Torrance DS, Stocking KL, Glaccum MB, Otten C, Willis R *et al.* TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. *J Immunol* 1998; **160**: 943–952.
5 Eliçabe RJ, Cargnelutti E, Serer MI, Stege PW, Valdez SR, Toscano MA *et al.* Lack of TNFR p55 results in heightened expression of IFN-γ and IL-17 during the development of reactive arthritis. *J Immunol* 2010; **185**: 4485–4495.
6 Nelson BH. IL-2, regulatory T cells, and tolerance. *J Immunol* 2004; **172**: 3983–3988.
7 Zheng SG, Wang JH, Gray JD, Soucier H, Horwitz DA. Natural and induced CD4$^+$ CD25$^+$ cells educate CD4$^+$CD25$^-$ cells to develop suppressive activity: the role of IL-2, TGF-β, and IL-10. *J Immunol* 2004; **172**: 5213–5221.
8 Chen X, Bäumel M, Männel DN, Howard OM, Oppenheim JJ. Interaction of TNF with TNF receptor type 2 promotes expansion and function of mouse CD4+CD25+ T regulatory cells. *J Immunol* 2007; **179**: 154–161.
9 Biton J, Semerano L, Delavallée L, Lemeiter D, Laborie M, Grouard-Vogel G *et al.* Interplay between TNF and regulatory T cell in a TNF-driven murine model of arthritis. *J Immunol* 2011; **186**: 3899–3910.
10 Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor Foxp3. *Immunity* 2005; **22**: 329–341.
11 Sfikakis PP. The first decade of biologic TNF antagonists in clinical practice: lessons learned, unresolved issues and future directions. *Curr Dir Autoimmun* 2010; **11**: 180–210.
12 Jacob CO, McDevitt HO. Tumor necrosis factor-alpha in murine autoimmune 'lupus' nephritis. *Nature* 1988; **331**: 356–358.
13 Yang XD, Tisch R, Singer SM, Cao ZA, Liblau RS, Schreiber RD *et al.* Effect of tumor necrosis factor-alpha on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process. *J Exp Med* 1994; **180**: 995–1004.
14 Liu J, Marino MW, Wong G, Grail D, Dunn A, Bettadapura J *et al.* TNF is a potent anti-inflammatory cytokine in autoimmune-mediated demyelination. *Nat Med* 1998; **4**: 78–83.
15 Biton J, Boissier MC, Bessis N. TNFα activator or inhibitor of regulatory T cells? *Joint Bone Spine* 2012; **79**: 119–123.
16 Murai M, Krause P, Cheroutre H, Kronenberg M. Regulatory T-cell stability and plasticity in mucosal and systemic immune systems. *Mucosal Immunol* 2010; **3**: 443–449.
17 Gaston JS, Cox C, Granfors K. Clinical and experimental evidence for persistent *Yersinia* infection in reactive arthritis. *Arthritis Rheum* 1999; **42**: 2239–2242.
18 Murai M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H *et al.* Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor FoxP3 and suppressive function in mice with colitis. *Nat Immunol* 2009; **10**: 1178–1184.
19 Grinberg-Bleyer Y, Saadoun D, Baeyens A, Billiard F, Goldstein JD, Grégoire S *et al.* Pathogenic T cells have a paradoxical protective effect in murine autoimmune diabetes by boosting Tregs. *J Clin Invest* 2010; **120**: 4558–4568.
20 Housley WJ, Adams CO, Nichols FC, Puddington L, Lingenheld EG, Zhu L *et al.* Natural but not inducible regulatory T cells require TNF-α signaling for *in vivo* function. *J Immunol* 2011; **186**: 6779–6787.
21 Eliçabe RJ, Arias JL, Rabinovich GA, Di Genaro MS. TNFRp55 modulates IL-6 and nitric oxide responses following *Yersinia* lipopolysaccharide stimulation in peritoneal macrophages. *Immunobiology* 2011; **216**: 1322–1330.
22 Brackertz D, Mitchell GF, Mackay IR. Antigen-induced arthritis in mice, I: Induction of arthritis in various strains of mice. *Arthritis Rheum* 1977; **20**: 841–850.
23 Wang X, Ma Y, Yoder A, Crandall H, Zachary JF, Fujinami RS *et al.* T cell infiltration is associated with increased Lyme arthritis in TLR2$^{-/-}$ mice. *FEMS Immunol Med Microbiol* 2008; **52**: 124–133.
24 Sakaguchi N, Takahashi T, Hata H, Nomura T, Tagami T, Yamazaki S *et al.* Altered thyme T-cell selection due to a mutation of ZAP-70 gene causes autoimmune arthritis in mice. *Nature* 2003; **426**: 454–460.
25 Chaudhry A, Samstein RM, Treuting P, Liang Y, Pils MC, Heinrich JM *et al.* Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity* 2011; **34**: 566–578.
26 Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* 2009; **10**: 595–602.
27 Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, Kas A *et al.* CD4$^+$ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science* 2009; **326**: 986–991.
28 Ilarregui JM, Croci DO, Bianco GA, Toscano MA, Salatino M, Vermeulen ME *et al.* Tolerogenic signals delivered by dendritic cells to T cells through a galectin-1-driven immunoregulatory circuit involving interleukin 27 and interleukin 10. *Nat Immunol* 2009; **10**: 981–991.
29 Hoeve MA, Savage ND, de Boer T, Langenberg DM, Waal Malefyt R, de,. Ottenhoff TH *et al.* Divergent effects of IL-12 and IL-23 on the production of IL-17 by human T cells. *Eur J Immunol* 2006; **36**: 661–670.
30 Zakharova M, Ziegler HK. Paradoxical anti-inflammatory actions of TNF-alpha: inhibition of IL-12 and IL-23 via TNF receptor 1 in macrophages and dendritic cells. *J Immunol* 2005; **175**: 5024–5033.
31 Notley CA, Inglis JJ, Alzabin S, McCann FE, McNamee KE, Williams RO. Blockade of tumor necrosis factor in collagen-induced arthritis reveals a novel immunoregulatory pathway for Th1 and Th17 cells. *J Exp Med* 2008; **205**: 2491–2497.
32 Kim EY, Priatel JJ, Teh SJ, Teh HS. TNF receptor type 2 (p75) functions as a costimulator for antigen-driven T cell responses *in vivo*. *J Immunol* 2006; **176**: 1026–1035.
33 Annunziato F, Cosmi L, Liotta F, Lazzeri E, Manetti R, Vanini V *et al.* Phenotype, localization, and mechanism of suppression of CD4(+)CD25(+) human thymocytes. *J Exp Med* 2002; **196**: 379–387.
34 Chen X, Subleski JJ, Hamano R, Howard OM, Wiltrout RH, Oppenheim JJ. Co-expression of TNFR2 and CD25 identifies more of the functional CD4+FOXP3+ regulatory T cells in human peripheral blood. *Eur J Immunol* 2010; **40**: 1099–1106.
35 Chen X, Hamano R, Subleski JJ, Hurwitz AA, Howard OM, Oppenheim JJ. Expression of costimulatory TNFR2 induces resistance of CD4+FoxP3- conventional T cells to suppression by CD4+FoxP3+ regulatory T cells. *J Immunol* 2010; **185**: 174–182.
36 Di Genaro MS, Cargnelutti DE, Eliçabe JR, Lacoste MG, Valdez S, Gómez N *et al.* Role of TNFRp55 in *Yersinia enterocolitica* O:3-induced arthritis: triggering bacterial antigens and articular immune response. *Rheumatology (Oxford)* 2007; **46**: 590–596.
37 Manzur MJ, Muñoz RV, Lucero AA, Juri Ayub M, Alvarez SE, Ciuffo GM. Production of recombinant enzymes of wide use for research. *Electronic J Biotech* 2006; **9**: 291–296.
38 Kyo F, Futani H, Matsui K, Terada M, Adachi K, Nagata K *et al.* Endogenous IL-6, but not tumor necrosis factor alpha, contributes to the development of toll-like receptor 4/myeloid differentiation factor 88-mediated acute arthritis in mice. *Arthritis Reum* 2005; **52**: 2530–2540.