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TNFR1 signalling is a critical checkpoint for developing macrophages that control of T-cell proliferation

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Summary

Macrophages (Mφ) are professional antigen-presenting cells, but when they accumulate at sites of inflammation, they can inhibit T-cell proliferation. In experimental autoimmune uveoretinitis, this limits the expansion of T cells within the target organ. To define requirements for the elaboration of this outcome, we have generated populations of Mφ *in vitro* that could also regulate T-cell responses; stimulating $CD4^+$ T-cell activation and cytokine production, but simultaneously suppressing T-cell proliferation. When T cells are removed from the influence of such cells, normal T-cell responses are restored. We show that tumour necrosis factor 1 (TNFR1) signalling is a critical checkpoint in the development of such Mφ, as $TNFR1^{-/-}$ Mφ are unable to suppress T-cell proliferation. This deficit in antigen-presenting cells results in a lack of production of prostaglandin E_2 (PGE_2) and nitric oxide, which are critical effector mechanisms that inhibit T-cell division. However, TNFR1 signalling is not required for the inhibitory function of Mφ because we could circumvent the requirement for this receptor, by maturing Mφ in the presence of exogenous interferon-γ and PGE_2. This produced $TNFR1^{-/-}$ Mφ that inhibited T-cell proliferation and indicates that TNFR1 delivers a signal that is necessary for the development but not the execution of this function.

Keywords: autoimmunity; experimental autoimmune uveoretinitis; macrophage; nitric oxide; prostaglandin E_2

Introduction

Organ-specific autoimmune diseases, such as multiple sclerosis and inflammatory eye disease, are co-ordinated by the activation of autoantigen-specific T cells, which are recruited specifically to sites of disease.[1,2]. The release of inflammatory mediators leads to a leucocyte influx that consists of a complex mixture of cell types.[3,4] For example, at the peak of experimental autoimmune uveoretinitis (EAU), the murine model of human inflammatory eye disease, we observe a heterogeneous population of cells including $CD11b^+$ cells, which form the largest fraction of the immune cells present, with significant numbers of $CD4^+$ T cells and smaller numbers of $CD8^+$ T cells also detected.[5–7] In this environment, the large majority of $CD11b^+$ cells are usually described as macrophages (Mφ); they release inflammatory mediators and act as professional antigen-presenting cells (APCs).[8–10] They can stimulate autoantigen-specific $CD4^+$ T cells, by presenting MHC class II-restricted peptides and we have recently reported that Mφ derived from the inflamed retina of mice with EAU can act as myeloid regulatory cells, inhibiting T-cell proliferation while allowing normal antigen-specific T-cell cytokine production.[10]

One important cytokine produced by activated Mφ is tumour necrosis factor-α (TNF-α) and the expression of one of its receptors, TNFR1, is necessary for the normal development of organ-specific autoimmunity.[11,12] Blocking signals through this receptor produces a number of important changes in Mφ function, including the abro-

Abbreviations: APC, antigen-presenting cell; BM, bone marrow; COX, cyclo-oxygenase; c.p.m., counts per minute; EPR, E prostanoid receptor; IFN, interferon; L-NMMA, *N*(G)-mono-methyl-L-arginine; Mφ, macrophages; mAb, monoclonal antibody; MDSC, myeloid-derived suppressor cells; NO, nitric oxide; OVA, ovalbumin; PG, prostaglandin; SNAP, *S*-nitroso-*N*-acetyl-l,l-penicillamine; TNFR1, tumour necrosis factor receptor 1; v/v, volume/volume.

gation of nitric oxide (NO) release following interferon-γ (IFN-γ) stimulation,[11] with a concomitant reduction in tissue damage. In murine EAU, the loss of TNFR1 signalling is also associated with a dramatic reduction in $CD11b^+$ cell trafficking to the target organ, but an increase in the relative proportion of $CD4^+$ cells within the target organ,[10] suggesting that the control of T-cell proliferation by myeloid $CD11b^+$ cells in EAU may be dependent on TNFR1 signalling. Furthermore, $CD11b^+$ cells from the eyes of $TNFR1^{-/-}$ mice immunized for EAU were able to stimulate T-cell proliferation, unlike similar cells from TNFR1 replete mice.[10] This TNFR1-dependent correlation between myeloid regulatory function and NO production is consistent with previous findings in the mouse and rat that have associated NO with the inhibition of T-cell proliferation.[13–15] However, it was not established whether the abrogation of NO production, consequent to the absence of TNFR1, was sufficient to explain the relative increase in $CD4^+$ T cells in EAU and the loss of regulatory function by target organ-infiltrating Mφ. In addition, Mφ-like cells, called MDSC,[16] isolated under other chronic inflammatory situations, particularly from tumours, and that suppress anti-tumour immune responses, have been described previously.[17–19] These cells exhibit a range of characteristics that are unlikely to be controlled directly by TNFR1 signalling.

Here we describe populations of Mφ, generated *in vitro*, which can regulate T-cell responses. We show that the critical requirement for TNFR1 expression and signalling relates to the development of a regulatory myeloid cell phenotype, rather than being required for this regulatory function. Therefore, restoring signals downstream of TNF-α signalling leads to the generation of TNFR1-deficient cells that are competent to inhibit T-cell proliferation. We identify two independent processes that result from TNFR1 signalling that together play a critical role in the control of T-cell responses by Mφ, which are mediated by IFN-γ and prostaglandin E_2 (PGE_2) respectively. In cells where the $TNFR1^{-/-}$ signalling pathway is inhibited, the absence of these signals prevents Mφ differentiation into myeloid cells that regulate T-cell proliferation.

Materials and methods

Mice and reagents

C57BL/6 mice were originally obtained from Harlan UK Limited (Oxford, UK), C57BL/6 TNFR1(p55)-deficient mice ($TNFR1^{-/-}$) were obtained from The Jackson Laboratory (Bar Harbor, ME), and C57BL/6 OT-II transgenic mice[20] expressing the T-cell receptor-specific for chicken ovalbumin$_{323–339}$ (OVA) and $I\text{-}A^b$ were a kind gift of Dr Steve Anderton (Department of Biological Sciences, University of Edinburgh, UK). All mice were housed under specific pathogen-free conditions with food and water continuously available. In all experiments, female mice aged between 6 and 8 weeks were used. Treatment of animals conformed to the regulations for animal research as set down by the Home Office, UK and also to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The $OVA_{323–339}$ (ISQAVHAAHAEINEAGR) peptide (Sigma, Poole, UK) was at least 95% pure as determined by HPLC. Complete tissue culture medium was Dulbecco's modified Eagle's minimum essential medium (without phenol red) supplemented with 10% volume/volume (v/v) fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine, 1 mM sodium pyruvate and 5 μM 2-mercaptoethanol (all from Invitrogen, Paisley, UK). Anti-IFN-γ neutralizing monoclonal antibody (mAb) was purified from culture supernatant of XMG1.2 cells, using protein G columns according to standard protocols. Soluble TNFR1 fusion protein (sTNFR1-Ig) was a kind gift from Geoff Hale (Therapeutic Antibody Group, University of Oxford, UK). All fluorochrome-conjugated anti-mouse mAbs and secondary detection reagents used were purchased from BD Biosciences (Oxford, UK). Biotinylated anti-CD3ζ was from Upstate (Watford, UK), and purified polyclonal rabbit anti-mouse EP1, EP2, EP3 and EP4 were from Cayman Chemicals (Ann Arbor, MI).

Preparation of cells

Bone marrow (BM) Mφ were generated using a method adapted from Munder *et al.*[21] Briefly, bone marrow cells were resuspended at 5×10^5 cells/ml in complete media supplemented with 5% v/v horse serum (Invitrogen), and 50 pg/ml macrophage colony-stimulating factor. The cell suspension was transferred to hydrophobic PTFE-coated tissue culture bags (supplied by Dr M. Munder, University of Heidelberg, Heidelberg, Germany) and incubated for 8 days at 37° in 5% v/v CO_2.

Single-cell splenocyte suspensions were generated by grinding spleens through a 70-μm cell strainer (BD Biosciences) with a syringe plunger. When used as APCs, splenocytes were irradiated with 3000 Rads using a caesuim-137 source (Gravatom, Hants, UK).

The OT-II $CD4^+$ T cells were prepared by enriching $CD4^+$ cells from single cell suspensions of C57BL/6 OT-II splenocytes, using anti-CD4 microbeads (Miltenyi Biotech, Bisley, UK) according to the manufacturer's instructions. B cells were prepared from spleens using anti-B220 microbeads (Miltenyi Biotech). Dendritic cells were generated from cultures of bone marrow cells as previously described.[22]

Cell culture

The 1×10^5 APCs were co-cultured with $CD4^+$ T cells at ratio of 1 : 1 in round-bottom 96-well plates in complete media. The OVA peptide was added at the indicated

concentrations. To some cultures the arginine analogue, L-N^G-monomethyl arginine, the NO donor *S*-nitroso-*N*-acetyl-l,l-penicillamine, or the cyclo-oxygenase (COX) inhibitor indomethacin (all from Sigma) was added. In some experiments, recombinant IFN-γ (Peprotech, London, UK), or PGE_2 (Sigma) was added. Cells were cultured in a humidified environment at 37°, 5% v/v CO_2. Proliferation was measured by pulsing with 18·5 kBq [^{3}H]thymidine (GE Healthcare, Bucks, UK) per well for the final 8 hr of culture and determining thymidine uptake [measured in counts per minute (c.p.m.)]. Accumulated NO production was measured after 64 hr in culture supernatants using Griess reagent (Sigma) as previously described.[23] Production of IFN-γ was assessed using a murine T helper type 1 (Th1)/Th2 Flow cytomix 10plex kit (Bender Medsystems, Vienna, Austria) according to the manufacturer's instructions. Concentration of PGE_2 was measured using an enzyme immunoassay competition enzyme-linked immunosorbent assay kit (Caymen Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Flow cytometry

Cell suspensions were incubated with 24G2 cell supernatant for 5 min at room temperature before incubation with primary mAb at 4° for 20 min. Intracellular staining was carried out using a cytofix/cytoperm kit according to

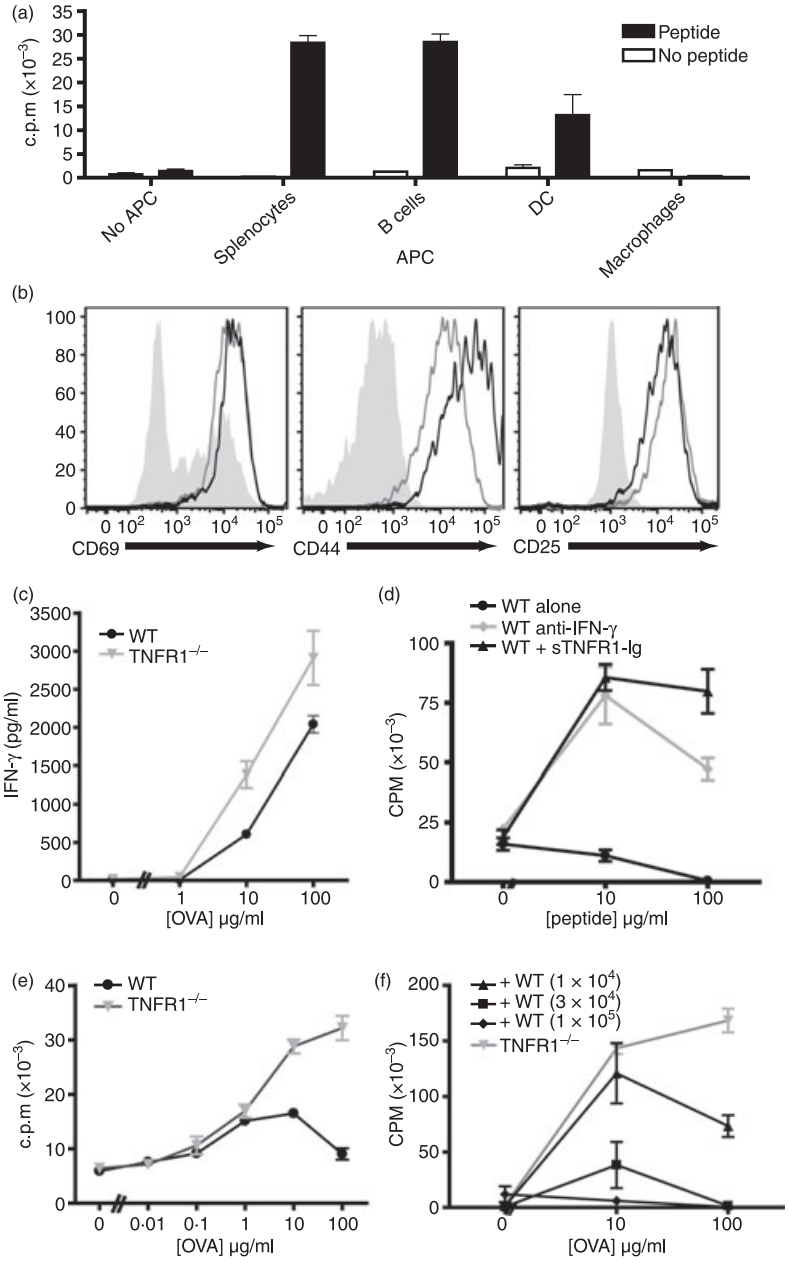

Figure 1 Antigen presentation by macrophages activates both T cells and macrophages, and inhibits T cell proliferation in a tumour necrosis factor-α (TNF-α) dependent manner. Ovalbumin (OVA) -specific OT-II $CD4^+$ T cells were purified and co-cultured at a 1 : 1 ratio with a variety of syngeneic antigen-presenting cells (APCs) for 72 hr in the presence or absence of 10 μg/ml OVA peptide. The APCs tested were whole splenocytes, purified B cells, and dendritic cells (DCs) and macrophages (Mφ) derived *ex vivo* from bone marrow cells. T-cell activation assessed by proliferation (a). The activation of $CD4^+$ cells co-cultured with Mφ (black lines), or with splenocytes (grey lines) is similar compared with naive $CD4^+$ cells (filled grey) (b). Wild-type (WT) or tumour necrosis factor receptor 1 deficient ($TNFR1^{-/-}$) Mφ elicit similar levels of interferon-γ (IFN-γ) production by T cells 72 hr after activation (c). Mφ-dependent inhibition of T-cell proliferation is prevented by blocking with anti-IFN-γ neutralizing monoclonal antibody (10 μg/ml) (grey circles) or sTNFR1-immunoglobulin fusion protein (10 ng/ml) (black triangles) compared with untreated control cultures (black circles). (d). OT-II T cells were co-cultured with $TNFR1^{-/-}$ Mφ or WT Mφ across a range of peptide concentrations (e). OT-II T cells were co-cultured with $TNFR1^{-/-}$ Mφ (1×10^5 cells/well) and WT Mφ in increasing numbers as indicated (f). These data are representative of three independent experiments.

the manufacturer's instructions (BD Biosciences). Cell suspensions were acquired with an LSR-II flow cytometer (BD Cytometry Systems). Analysis was carried out using FlowJo software (TreeStar, San Carlos, CA).

Statistical analyses

Using Prism 4 software (GraphPad Software Inc., San Diego, CA), comparisons of statistical significance between groups were assessed using the Mann–Whitney *U*-test.

Results

In inflammatory environments, recruited leucocytes may have emergent properties that are dependent on multiple local interactions with many different soluble signalling molecules. In EAU, accumulating Mφ, derived from BM cells, infiltrate inflammatory sites in large numbers and perform as professional APCs. They interact with T cells, both enhancing and regulating immunity. We have demonstrated that the Mφ that accumulate in the target organ modify T cell responses, suppressing T cell proliferation but preserving cytokine secretion.[10] These Mφ express cell surface markers such as Gr1 and CD31 that are associated with immune regulation, and to investigate the function of such cells, we generated Mφ *in vitro* from BM cells cultured in an inert environment (hydrophobic PTFE-coated tissue culture bags). We compared the ability of these cells to present antigen with other APCs. The $OVA_{323–339}$-specific TCR transgenic OT-II $CD4^+$ T cells were co-cultured with different populations of professional APCs in the presence or absence of cognate OVA peptide. Wild-type (WT) splenocytes, B cells and dendritic cells stimulated peptide-specific T-cell proliferation, but BM-Mφ did not (Fig. 1a).

To address whether this was the result of a failure of Mφ to interact with T cells, we analysed other markers of T-cell activation. Despite the lack of proliferation, we observed that, following co-culture with BM-Mφ, OT-II T cells adopted an activated cell surface phenotype and expressed high levels of CD69, CD44 and CD25 (Fig. 1b). The OT-II T cells activated by Mφ also produced high levels of IFN-γ, the production of which was shown to be independent of TNFR1 signalling as BM-Mφ derived from TNFR1 knockout ($TNFR1^{-/-}$) mice stimulated T cells to produce similar amounts of IFN-γ. Interferon-γ activates Mφ, which in turn leads to autocrine TNF-α signalling that further mediates Mφ activation.[11] Blocking Mφ activation by neutralizing IFN-γ or TNF-α by the addition of anti IFN-γ mAb or sTNFR1-immunoglobulin fusion protein restored peptide-dependent T-cell proliferation (Fig. 1d), supporting our previous data that the regulation of T-cell proliferation by myeloid cells in the target organ during autoimmunity is dependent on the activation of myeloid cells by IFN-γ and TNF-α.[10] Consistent with these *in vitro* blocking studies, $TNFR1^{-/-}$ Mφ stimulated T-cell proliferation across a range of peptide concentrations, whereas WT Mφ stimulated little proliferation (Fig. 1e). Therefore, both WT and $TNFR1^{-/-}$ can activate T cells in a peptide-dependent manner, but WT Mφ do not induce T-cell proliferation.

We then addressed whether WT Mφ inhibition of T-cell proliferation was a dominant effect. Addition of increasing numbers of WT Mφ to cultures where OT-II T cells were activated by $TNFR1^{-/-}$ Mφ led to a dose-dependent inhibition of proliferation. Adding WT Mφ at a ratio of 1 : 1 with the $TNFR1^{-/-}$ Mφ, prevented the proliferation induced by $TNFR1^{-/-}$ Mφ (Fig. 1f). This

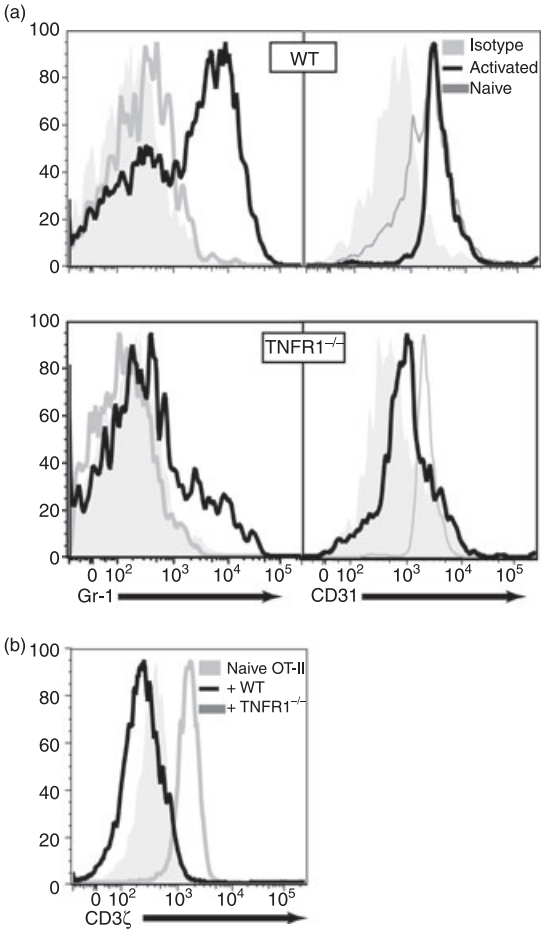

Figure 2. Tumour necrosis factor receptor 1 deficient ($TNFR1^{-/-}$) macrophages have a less inhibitory phenotype than wild-type (WT) macrophages. WT or $TNFR1^{-/-}$ bone-marrow derived macrophages (BM-Mφ) were co-cultured with OT-II T cells with 10 μg/ml ovalbumin (OVA) for 72 hr, then surface stained CD4, CD11b, CD31 and Gr-1 (a) and stained intracellularly with anti-TCRξ monoclonal antibody (b). Gr-1 up-regulation was greater with WT Mφ while CD3ξ expression was reduced compared with naive OT-II $CD4^+$ T cells (grey filled) and OT-II $CD4^+$ T cells co-cultured for 72 hr with $TNFR1^{-/-}$ Mφ (grey lines). These data are representative of three independent experiments.

TNF-α-dependent suppression of T-cell proliferation by naive Mφ is similar to that induced by Mφ in autoimmunity and by populations of myeloid-derived suppressor cells (MDSC), which prevent T-cell responses in tumour sites.[13,16]

The Mφ from sites of autoimmune inflammation and MDSC share phenotypic markers, including the expression of CD11b, Gr-1 and CD31, which have been useful in identifying myeloid cells that can inhibit T-cell proliferation. As a consequence, we examined the phenotype of *in vitro*-generated naive Mφ and observed that, consistent with *in vivo*-generated Mφ, they expressed CD11b, CD31 and F4/80, but not Gr-1 (Supplementary Fig. S1). The activation of BM-Mφ with LPS or IFN-γ, in the absence of T cells, did not lead to the expression of Gr-1 (data not shown). However, when BM-Mφ were activated by co-culture with T cells and cognate peptide, both WT and TNFR1$^{-/-}$ Mφ up-regulated Gr-1 (Fig. 2a), indicating a requirement for signals supplied by T cells for Gr-1 expression. Naive Mφ from either mouse strain expressed CD31, which was down-regulated to a greater extent on TNFR1$^{-/-}$ Mφ compared with WT Mφ following activation (Fig. 2a). Interestingly, the mechanism by which Mφ acquire a suppressive Gr-1^{+} phenotype appears to require cell–cell contact with activated T cells, rather than resulting from stimulation by soluble factors (Supplementary Fig. S2).

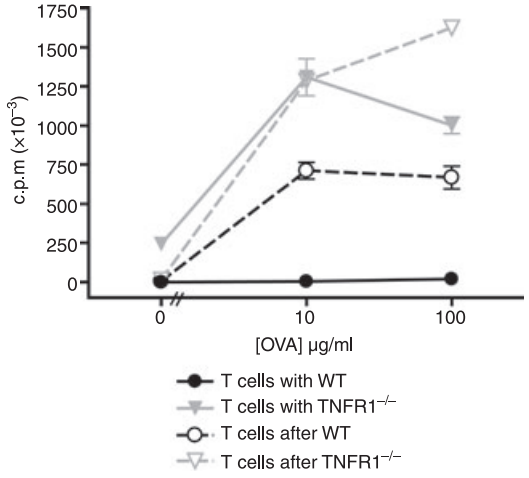

Figure 3. T cells regain the ability to proliferate when removed from macrophages. Wild-type (WT) or tumour necrosis factor receptor 1 deficient (TNFR1$^{-/-}$) bone-marrow-derived macrophages (BM-Mφ) were co-cultured with OT-II T cells in the presence of ovalbumin (OVA) peptide. After 24 hr, non-adherent cells were transferred to fresh plates (re-plated) and all cells were washed into fresh media. Proliferation was measured after a further 48 hrs of culture for control cells (solid lines) and re-plated cells (broken lines) for cultures initially containing either WT macrophages (black lines) or TNFR1$^{-/-}$ macrophages (grey lines). These data are representative of four independent experiments.

The inhibition of T-cell proliferation in the presence of tumour-derived Mφ has been associated with down-regulation of the ζ-chain of the CD3/TCR signal transduction complex.[10,24] To determine the effects on the intracellular expression of CD3ζ by OT-II CD4^{+} cells, we examined cells stimulated by WT or TNFR1$^{-/-}$ BM-Mφ. Compared with unstimulated T cells, activation with WT Mφ led to lower levels of CD3ζ (Fig. 2b) consistent with T-cell inhibition,[25] whereas activation with TNFR1$^{-/-}$ Mφ led to CD3ζ up-regulation, consistent with normal activation[26] (Fig. 2b).

Since Mφ in the local environment stimulate lymphocyte cytokine production but block the proliferation of T cells, we wished to ascertain the fate of T cells that escape

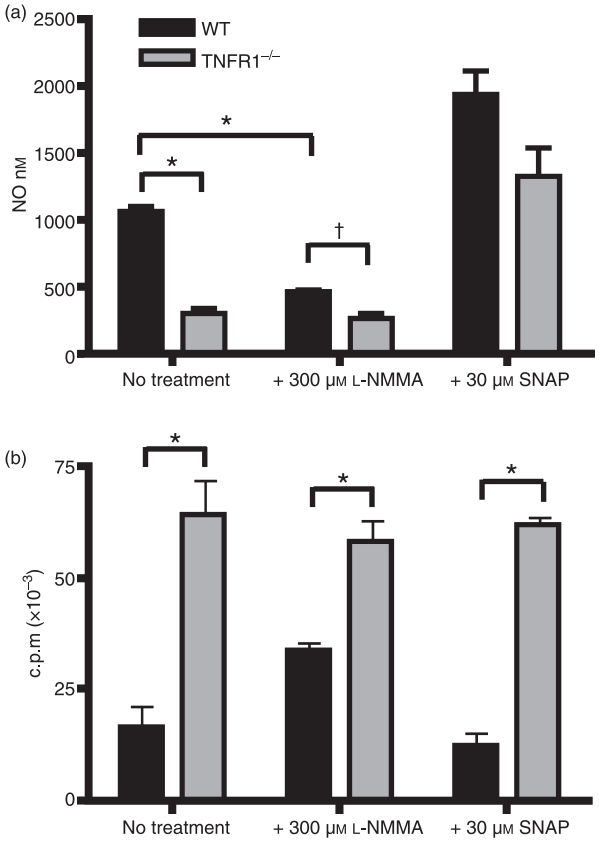

Figure 4. Regulation of T-cell proliferation is reversible and is partially mediated by nitric oxide (NO). Wild-type (WT) or tumour necrosis factor receptor 1 deficient (TNFR1$^{-/-}$) bone-marrow-derived macrophages (BM-Mφ) were co-cultured with OT-II T cells in the presence of 100 μg/ml ovalbumin (OVA) peptide for 72 hr. To some cultures, 300 μM *N*(G)-mono-methyl-L-arginine (L-NMMA), an arginine analogue, or 30 μM *S*-nitroso-*N*-acetyl-l,l-penicillamine (SNAP), a nitric oxide (NO) donor, was added. Plots show the effect of addition of L-NMMA or SNAP on NO production (a) and proliferation (b) on co-cultures containing WT (black bars) or TNFR1$^{-/-}$ (grey bars) Mφ, as compared with control untreated co-cultures. These data are representative of four independent experiments. *$P < 0{\cdot}01$; †not significantly different.

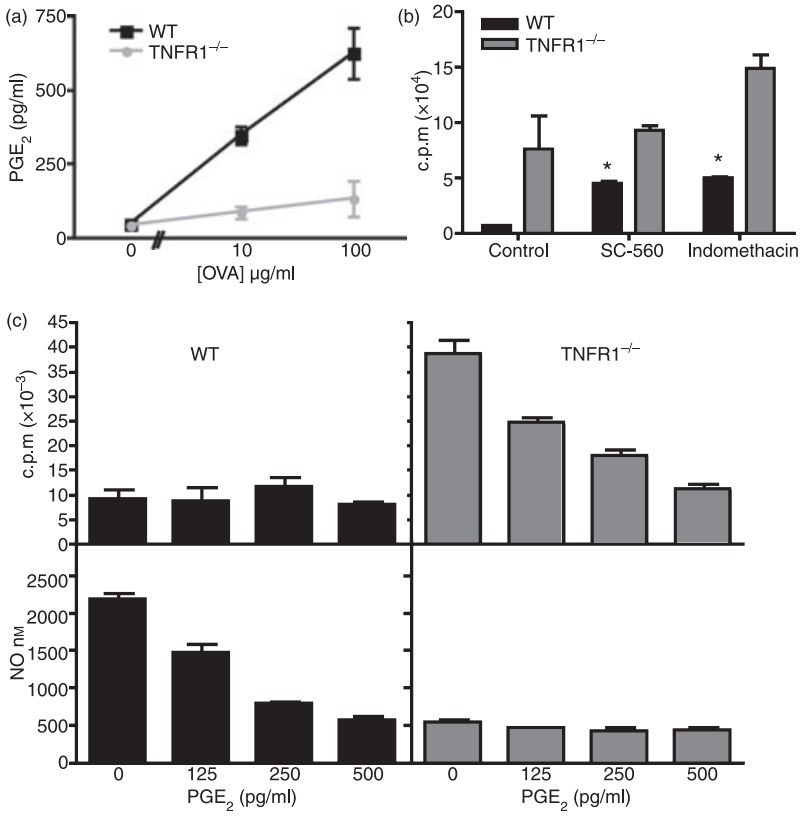

Figure 5. Signalling via tumour necrosis factor receptor 1 (TNFR1) enhances prostaglandin E_2 (PGE_2) production, which contributes to T-cell regulation. Wild-type (WT) or $TNFR1^{-/-}$ bone-marrow-derived macrophages (BM-Mφ) were co-cultured with OT-II T cells and ovalbumin (OVA) peptide for 72 hr. In some experiments the pan-cyclo-oxygenase (COX) inhibitor indomethacin or the COX-2-specfic inhibitor SC-256 was added. In other experiments, PGE_2 was added. PGE_2 concentration (a) and proliferation (b) of co-cultures containing WT Mφ (black bars) or $TNFR1^{-/-}$ Mφ (grey lines) treated with COX inhibitors was measured. Proliferation (top) and nitric oxide (NO) production (bottom) were measured from co-cultures with WT Mφ (black bars, left-hand graphs) or $TNFR1^{-/-}$ (grey bars, right-hand graph) with PGE_2 added at a range of concentrations (c). These data are representative of two independent experiments. *Significantly different to control WT Mφ $P < 0{\cdot}05$.

from their presence. To do this, we tested whether co-culture with inhibitory BM-Mφ produced a long-term unresponsive state in the T cells. OT-II $CD4^+$ T cells were combined with BM-Mφ and OVA peptide for 24 hr and then the non-adherent lymphocytes were removed and the T cells were re-plated in fresh medium. Cell proliferation was then assessed by [^{3}H]thymidine incorporation. OT-II T cells that had been stimulated by $TNFR1^{-/-}$ Mφ were able to proliferate and this capacity was maintained when they were removed from the APCs. Continuous culture of T cells with WT Mφ prevented proliferation, but in contrast, when the T cells were removed from the WT Mφ they were able to proliferate without further antigenic stimulation (Fig. 3). These data show that antigen presentation by Mφ to T cells for 24 hr produces a T cell that is poised to divide, but is held in check by factors in the local microenvironment.

Inhibition of T-cell proliferation by tumour-derived MDSC and inflammatory monocytes in experimental autoimmune encephalomyelitis has been reported to be the result of the production of NO.[27,28] Since $TNFR1^{-/-}$ BM-Mφ do not produce NO in response to IFN-γ *in vitro*, we wanted to test whether this deficiency was sufficient to explain the WT inhibition of T-cell proliferation, by restoring NO levels in the presence of $TNFR1^{-/-}$ BM-Mφ. In cultures of OT-II T cells with either WT or $TNFR1^{-/-}$ Mφ, we could significantly reduce NO production from WT BM-Mφ with the inhibitor *N*(G)-monomethyl-L-arginine (L-NMMA), or raise NO levels to concentrations above those produced by WT BM-Mφ with the NO donor *S*-nitroso-*N*-acetyl-l,l-penicillamine (SNAP) (Fig. 4a). Co-cultures of OT-II T cells and WT Mφ that were treated with a concentration of L-NMMA that reduced NO production to the levels observed in cultures with $TNFR1^{-/-}$ Mφ (Fig. 4a and Supplementary Fig. S3) only partially restored proliferation (Fig. 4b). Furthermore, levels of NO that were associated with reduced T-cell proliferation in the context of WT BM-Mφ, were not sufficient to inhibit the proliferation induced by $TNFR1^{-/-}$ BM-Mφ (Fig. 4b and Supplementary Fig. S3). Therefore, although some T-cell suppression is the result of the presence of NO, NO alone is not sufficient to produce the complete spectrum of inhibitory effects induced by WT Mφ.

We then investigated other mechanisms by which Mφ can regulate T-cell responses. The soluble factor PGE_2 is produced by Mφ in response to TNF-α[29] and we found that culture of OT-II T cells with WT Mφ in the presence of cognate peptide led to high levels of PGE_2, whereas similar culture with $TNFR1^{-/-}$ Mφ did not (Fig. 5a). As PGE_2 has previously been associated with the differentiation of myeloid cells that inhibit T-cell responses in tumours,[30] we examined whether its presence was a significant factor in the inhibition of T-cell proliferation by BM-Mφ. We inhibited PGE_2 production with COX inhibitors (SC-560, a COX-1 inhibitor, or indomethacin, a

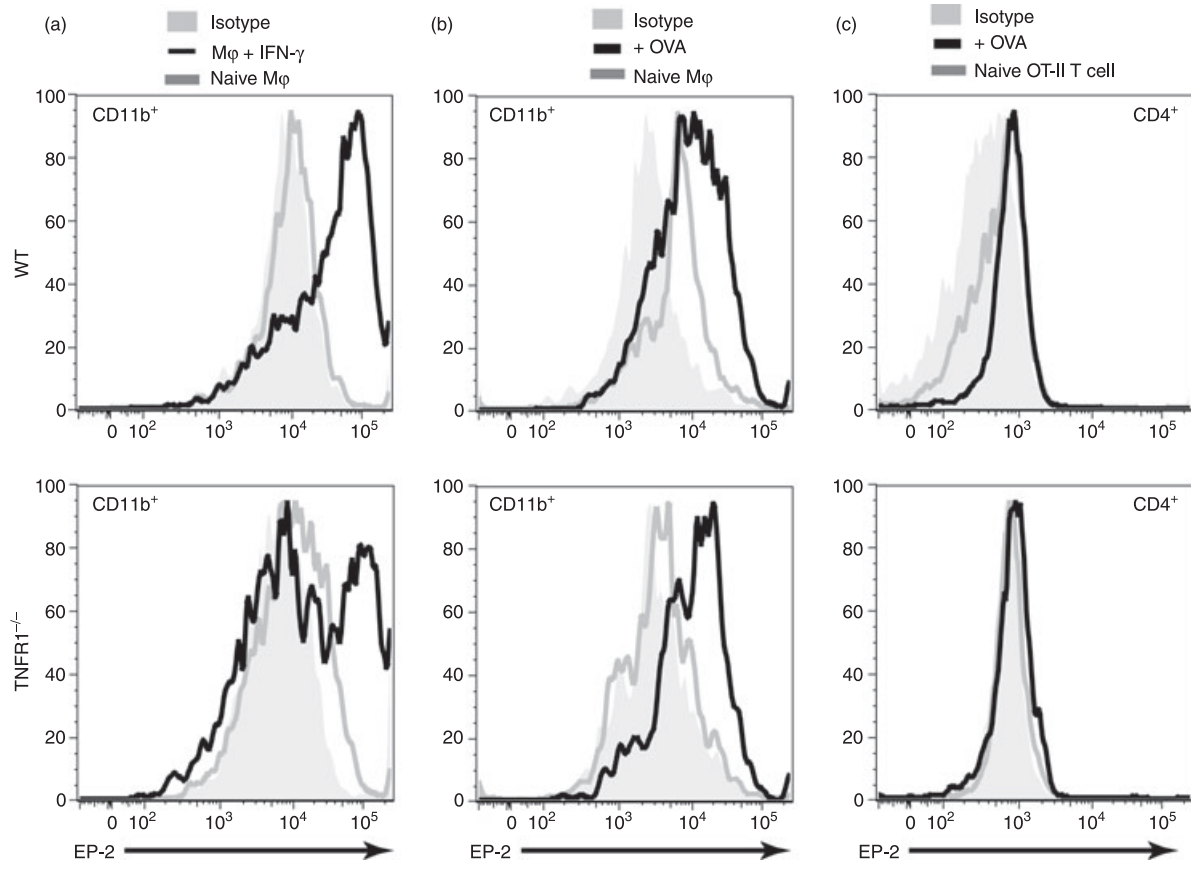

Figure 6. Interferon-γ (IFN-γ) induces E prostanoid (EP) receptor up-regulation on macrophages (Mφ). Wild-type (WT) or tumour necrosis factor receptor 1 deficient (TNFR1$^{-/-}$) Mφ were treated with 100 U/ml IFN-γ for 24 hr, or cultured with OT-II T cells in the presence of 10 μg/ml ovalbumin (OVA) for 72 hr, and stained with monoclonal antibodies (mAbs) against CD11b and EP-2. EP-2 expression by IFN-γ-activated CD11b$^+$ cells (a), naive OT-II CD4$^+$ cells or cells activated by Mφ (b) or CD11b$^+$ cells from the same co-cultures (c) are shown. Isotype controls (grey-filled) and corresponding naive cells (grey lines) is shown. These data are representative of two independent experiments.

pan-COX inhibitor), which restored OT-II T-cell proliferation (Fig. 5b) to levels that were a third to a half as great as those induced by TNFR1$^{-/-}$ Mφ. The addition of exogenous PGE_2 led to a dose-dependent reduction in OT-II T-cell proliferation stimulated by TNFR1$^{-/-}$ Mφ (Fig. 5c), and also inhibited WT NO production from WT Mφ in co-culture.

The effects of PGE_2 are mediated through one or more of the four E prostanoid (EP) receptors, EP1, EP2, EP3 and EP4.[31] To determine which population of cells PGE_2 is acting on, we examined the expression of EP receptors on OT-II T cells and Mφ. Naive BM-Mφ expressed low levels of EP2 receptor, but stimulation by IFN-γ led to rapid up-regulation of EP2 by both WT and TNFR1$^{-/-}$ Mφ, although this up-regulation was greater on WT cells (Fig. 6a). A similar up-regulation was observed when WT or TNFR1$^{-/-}$ Mφ were activated by co-culture with OT-II T cells and cognate peptide (Fig. 6b). In contrast, OT-II T cells expressed little or no EP2 receptor either when naive or when activated by cognate OVA peptide presented by either WT or TNFR1$^{-/-}$ Mφ (Fig. 6c). Similar results were obtained for other EP receptors, EP1, EP3 and EP4 (data not shown). These data indicated that, unlike PGE_2 (and NO) production, EP receptor up-regulation was independent of TNF-α signalling and that PGE_2 in this system most likely acts through effects on Mφ.

As EP receptor up-regulation was IFN-γ dependent, but TNFR1 independent (Fig. 6a), we reasoned that the up-regulation of these receptors might poise the Mφ to receive an autocrine PGE_2 signal the induction of which was TNFR1 dependent. If this were the case, and if TNFR1 signalling was critical in maturing inhibitory Mφ but not needed for their function, then treatment with a combination of IFN-γ and PGE_2 should circumvent the lack of TNFR1 signalling in TNFR1$^{-/-}$ Mφ. To test this TNFR1$^{-/-}$ BM-Mφ were pre-incubated for 72 hr with a combination of PGE_2 and IFN-γ separately or together. These treatments did not result in an up-regulation of Gr-1. Nevertheless, the use of the combination of reagents, but not either reagent alone, produced a TNFR1$^{-/-}$ Mφ that could both inhibit T-cell proliferation (Fig. 7a) and produce NO (Fig. 7b).

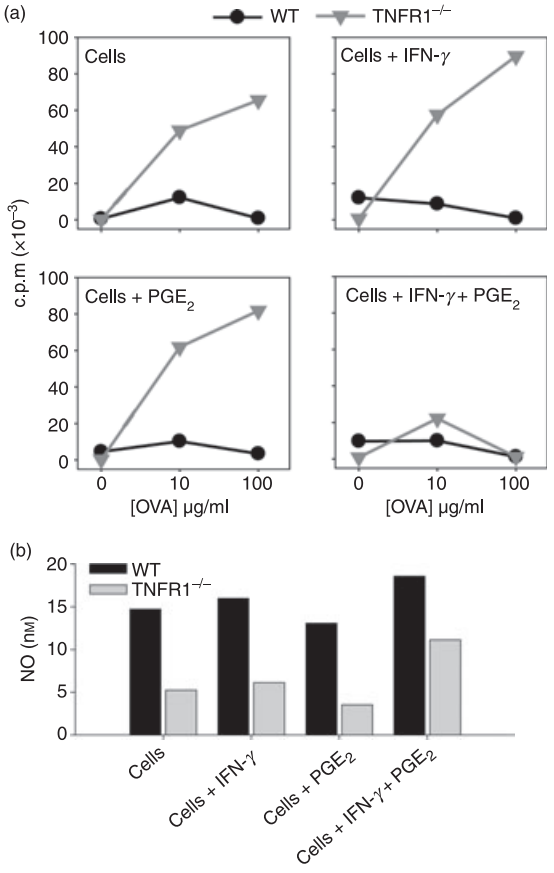

Figure 7. Treatment with interferon-γ/prostaglandin E_2 (IFN-γ/PGE_2) circumvents the requirement for tumour necrosis factor receptor 1 (TNFR1) signalling in the generation of regulatory macrophages. Wild-type (WT) or $TNFR1^{-/-}$ bone-marrow-derived macrophages (BM-Mφ) were incubated in PTFE-coated plates for 72 hr in the presence of recombinant IFN-γ (100 U/ml), recombinant PGE_2 (1 ng/ml), or both together. After 72 hr, washed cells were co-cultured with OT-II T cells in the presence of OVA peptide (100 μg/ml) for a further 72 hr when T-cell proliferation (a) and nitric oxide (NO) elicited (b) were measured. Pre-treatment with IFN-γ and PGE_2 together produces $TNFR1^{-/-}$ Mφ that suppress proliferation. These data are representative of three independent experiments.

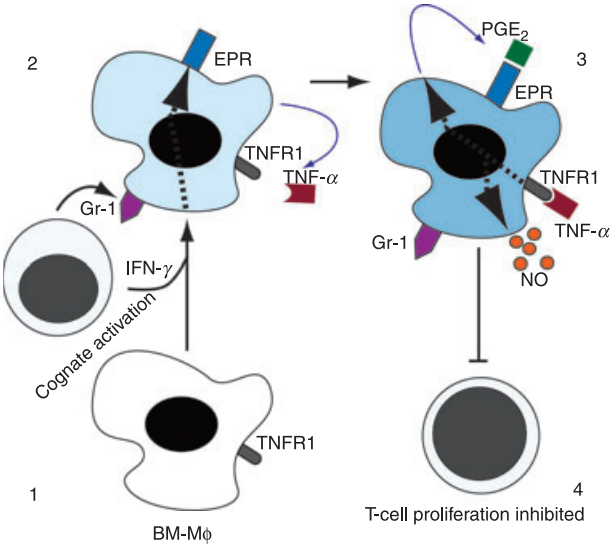

Figure 8. Maturation of bone-marrow-derived macrophages (BM-Mφ) to produce a suppressive antigen-presenting cell (APC). BM-Mφ that receive signals via cell contact and interferon-γ/ (IFN-γ) (1) up-regulate Gr-1 and E prostanoid receptor (EPR) and produce autocrine tumour necrosis factor-α (TNF-α) (2). TNF-α signalling stimulates prostaglandin E_2 (PGE_2) and nitric oxide (NO) production by the Mφ (3) leading it to generate a local microenvironment that permits T-cell activation but inhibits T-cell proliferation (4).

Discussion

In this paper, we explore the role that TNFR1 signalling plays in inducing myeloid cells that can selectively limit T-cell growth. Cognate interactions between T cells and Mφ that lack TNFR1 lead to activation marker up-regulation, cytokine production and T-cell proliferation, whereas the interaction between the similar T cells and WT BM-Mφ results in activation marker up-regulation and cytokine production, but not in T-cell division. We have shown that peptide presentation by WT or $TNFR1^{-/-}$ Mφ to OT-II T cells is sufficient to induce IFN-γ, and that IFN-γ alone can stimulate the up-regulation of EP receptors on WT and $TNFR1^{-/-}$ Mφ (Figs 6 and 8). This up-regulation is induced more efficiently in the presence of T cells and, furthermore, cell–cell interactions are required for the up-regulation of Gr-1, which accompanies the differentiation to a suppressive phenotype *in vivo* (Fig. 8).

Interferon-γ also drives the production of low levels of TNF-α that are sufficient to stimulate BM-Mφ to produce PGE_2 and NO in an autocrine manner (Fig. 8). Both of these effector molecules contribute to the subsequent cell cycle arrest in neighbouring T cells; an inhibition that is reversible if the T cells are removed from the vicinity of the Mφ and washed (Fig. 3).

The ability of Mφ to reduce T-cell responses has been documented for many years.[32] In tumour models, this is thought to contribute to tumour escape from immunosurveillance, but it is unlikely that this represents a normal physiological expression of this process. In inflammation stimulated by infection, restricting T-cell proliferation within the tissue could have a role simply by sparing finite metabolic resources for other effector cells that are present. Rapid T-cell division is highly dependent on local glucose[33] and activated Mφ also consume glucose and other sources of metabolic energy at a high rate.[34,35] Therefore, limiting proliferation may be a form of immune system triage at the site of inflammation. Another possibility is that restricting T-cell activation prevents the differentiation of antigen-specific T cells within tissues. Segregating the environment in which T

cells differentiate, from that in which they exercise effector function, could reduce the generation of T-cell effector cells that can be activated by autoantigens. At a site of acute inflammation, Mφ will be processing large amounts of damaged normal tissue that might lead to an increased risk of local autoimmunity.

It is not, however, the case that T-cell immunity is entirely shut down in this inflammatory microenvironment. Our demonstration that T cells removed from the presence of Mφ can resume proliferation (Fig. 2) shows that T cells that traffic away from the inflammatory environment will still be able to contribute to the pool of circulating activated antigen-specific cells. This local immune response could still serve to amplify T-cell responses and support the production of immunological memory.

In terms of Mφ function, our data suggest that a lack of TNFR1 signalling impedes the development of Mφ with the capacity to inhibit T cells. This critical role for TNFR1 in the generation of these cells also suggests TNFR1 may be important to the generation of MDSC in tumours. Therefore, our study throws light on other previously unexplained findings: that in a model of metastasizing lung carcinoma, although tumours initially expand at normal rates, in TNFR1$^{-/-}$ mice, metastases regress after 21 days.[36] Also in TNFR1$^{-/-}$ mice and mice treated with TNFR1$^{-/-}$ bone marrow,[37] there is a reduced tumour burden in a model of colorectal carcinoma. We suggest that this may relate to a failure to generate functional MDSC. However, other factors also remain important, because the efficacy of TNF-α blockade, which has been used as a therapy in late-stage ovarian carcinoma, maps at least partially to a defect in TNFR1 signalling to T cells.[38]

The lack of TNFR1 was also associated with a lack of PGE_2 production. It has been previously demonstrated that PGE_2 is required for MDSC maturation *in vivo*.[30,39] PGE_2 can also modulate the function of dendritic cells as APCs, and this effect depends on expression of EP2 or EP4 by the dendritic cell.[40] It is also important to note that certain aspects of Mφ activation (specifically up-regulation of Gr-1) are dependent on cell–cell contact and not mediated by soluble factors (Fig. 2 and supplementary Fig. S2). Up-regulation of Gr-1 is not part of the maturation process demonstrated in Fig. 7, and although a role in limiting T-cell proliferation is not ruled out by this experiment, the soluble mediators NO and PGE_2 together are sufficient to restrict T-cell proliferation.

Finally, it was striking that despite the strong phenotype of TNFR1$^{-/-}$ Mφ *in vitro* and *in vivo*, we could restore near normal inhibitory function by treating with a combination of soluble mediators (Fig. 7). This result illustrates on the one hand the emergent properties that multiple signals can have on cell function and on the other hand the many levels of redundancy that are inherent in Mφ responses. This redundancy complicates the analysis of function *in vitro* and *in vivo*, but it is also likely to produce immune responses that in the wild are more robust and less susceptible to a single targeted attack by a pathogen.

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Disclosures

The authors declare no competing interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Wild-type bone-marrow-derived macrophages (WT BM-Mφ) were prepared as described in the Materials and methods. Cells were then stained with antibodies against CD11b, CD31, F4/80, and Gr-1. Plot A shows F4/80 and CD11b expression by naive BM-Mφ. Plots B and C show CD31 and Gr-1 expression naive BM-Mφ (black lines) compared with isotype controls (grey filled).

Figure S2. Wild-type (WT) or tumour necrosis factor receptor 1 deficient (TNFR1$^{-/-}$) bone-marrow-derived macrophages (BM-Mφ) were co-cultured with OT-II T cells in the lower chambers of 0.22 μm transwells in the presence of 100 μg/ml ovalbumin (OVA) peptide. Equal numbers of either WT or TNFR1$^{-/-}$ BM-Mφ were added to the top chamber. After 72 hr, from the both chambers were harvested separately and stained with antibodies against CD11b and Gr-1 for flow cytometric analysis. Plots show Gr-1 expression of CD11b$^+$ cells, with Mφ from the top chamber (red lines) and those from the lower chamber (blue lines).

Figure S3. Wild-type (WT) or tumour necrosis factor receptor 1 deficient (TNFR1$^{-/-}$) bone-marrow-derived macrophages (BM-Mφ) were co-cultured with OT-II T cells in the presence of 100 μg/ml ovalbumin (OVA) peptide for 72 hr. *N*(G)-Mono-methyl-L-arginine (L-NMMA) or *S*-nitroso-*N*-acetyl-l,l-penicillamine (SNAP) was added at the indicated concentrations. T-cell proliferation was measured over the final 8 hr of culture. Nitric oxide (NO) production was measured in the culture supernatant. Plots show the effect of addition of L-NMMA or SNAP on NO production and proliferation on co-cultures containing WT (black lines) or TNFR1$^{-/-}$ (grey lines) Mφ, as compared with control co-cultures in which Mφ alone were cultured.

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