TNFR1-Dependent Regulation of Myeloid Cell Function in Experimental Autoimmune Uveoretinitis

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Experimental autoimmune uveoretinitis is an autoimmune disease induced in mice, which involves the infiltration of CD11b+ macrophages and CD4+ T cells into the normally immune-privileged retina. Damage is produced in the target organ following the activation of Th1 and Th17 T cells and by the release of cytotoxic mediators such as NO by activated macrophages. The majority of immune cells infiltrating into the retina are CD11b+ myeloid cells, but, despite the presence of these APCs, relatively limited numbers of T cells are observed in the retina during the disease course. These T cells do not proliferate when leukocytes are isolated from the retina and restimulated in vitro, although they do produce both IFN-γ and IL-17. T cell proliferation was restored by depleting the myeloid cells from the cultures and furthermore those isolated myeloid cells were able to regulate the proliferation of other T cells. The ability of macrophages to regulate proliferation depends on activation by T cell-produced IFN-γ and autocrine TNF-α signaling in the myeloid cells via TNFR1. In the absence of TNFR1 signaling, relative T cell expansion in the retina is increased, indicating that regulatory myeloid cells may also act in vivo. However, TNFR1 signaling is also required for macrophages, but not T cells, to migrate into the target organ. Thus, in TNFR1 knockout mice, the amplification of autoimmunity is limited, leading to resistance to experimental autoimmune uveoretinitis induction.


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E xperimental autoimmune uveoretinitis (EAU) is a murine model of the human autoimmune disease posterior uveitis. There is a close clinicopathologic correlation between EAU and aspects of human uveitis. This has allowed the successful dissection of immunopathologic mechanisms of autoimmune inflammation and tissue damage, and facilitated the development of immunotherapies (1–4). One successful immunotherapy both in preclinical models of uveitis and in human disease is the blockade of TNF-α (5).

EAU is initiated by the activation of CD4+ T cells that respond to ocular Ags located within or around photoreceptor segments (6–8). In mice, EAU is induced by administration of whole retinol binding protein (RBP)-3 (previously known as interphotoreceptor retinoid-binding protein), or immunodominant peptides derived from it, in an appropriate adjuvant (9). Infiltration by ocular Ag-specific T cells recruits macrophages into the eye and activates them, generating structural damage. At the peak of disease, the recruited macrophages greatly outnumber the resident microglia, and they are also present in greater numbers than the CD4+ T cells, although the proportion of CD4+ cells increases as the initial peak of disease resolves (10). The number of infiltrating cells closely correlates with clinical and histologic disease scores (11).

The activation of macrophages is an important determinant of disease outcome. Secretion of NO by retinal-infiltrating macrophages is critical for the generation of tissue-damaging superoxides, which ultimately leads to the loss of visual function (12). This activation is controlled by the microenvironment, and macrophages elaborate NO in response to a range of inflammatory stimuli, including IFN-γ (13). This response is also dependant on autocrine TNF-α production, and mice with a TNFR1 deletion have macrophages with a selective deficit in IFN-γ-dependant NO production and are resistant to the induction of EAU (14).

As well as initiating tissue damage, NO has also been shown to act on T cells, limiting their proliferative responses (15). This raised the question of whether or not NO-producing macrophages, present within the eye during EAU, limited local T cell proliferation and whether or not this regulation was TNFR1-dependent. In two different strains of mice, we found that EAU-inducing T cells, prepared from the retinal infiltrate, were unable to divide in the presence of infiltrating macrophages. This was due to active regulation of T cell proliferation by the infiltrating macrophages and was dependant on macrophage TNFR1 expression.

In vitro, TNFR1−/− macrophages support a greater level of T cell proliferation than wild-type (WT) macrophages. However, in vivo there is a reduction in the number of infiltrating cells which accumulate in the retina of TNFR1−/− mice following EAU induction and these mice are resistant to EAU. We investigated the factors limiting cell accumulation in the retina. We found a TNFR1-dependent deficit in macrophage, but not T cell, trafficking to the target organ. Our findings support the concept that targeting macrophage activation is a potentially effective approach to treating organ-specific autoimmunity.

Materials and Methods
Mice and reagents
B10.RIII and C57BL/6 were originally obtained from Harlan, C57BL/6 TNFR1p55-deficient mice (TNFR1−/−) were obtained from The Jackson Laboratory, and C57BL/6 Ly.5.2 congenic mice were obtained from...
Charles River Laboratory. All mice were housed under specific pathogen-free conditions with food and water continuously available. Female B10.RIII mice were aged between 6 and 8 wk at the time of disease induction. Treatment of animals conformed to the regulations for animal research as set down by the Home Office, U.K. and also to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. RBP-3(161–180) (SGIPYIISYLHPGNTILHVD) peptide was synthesized by Sigma Genosys to 95% purity. Complete tissue culture medium was DMEM (without phenol red) supplemented with 10% v/v FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 1 mM sodium pyruvate, and 5 μM 2-ME (all Invitrogen). All fluorochrome-conjugated anti-mouse Abs were purchased from BD Biosciences, biotinylated anti-CD3ε and pu- rified rat anti-CD11b (BD Biosciences) were from Upstate Biotech- nology. Secondary detection reagents used were PE-conjugated strep- vividin and FITC-conjugated goat anti-rabbit IgG (both BD Biosciences).

**EAU induction and generation of bone marrow chimeras**

Mice were immunized s.c. with peptides emulsified in CFA supplemented with Mycobacterium tuberculosis (MTb) complete H37 Ra (BD Bio- sciences). For B10.RIII mice, 50 μg RBP-3(161–180) and 125 μg MTb per mouse were used for C57BL/6 background mice, 500 μg RBP-3(161–180) and 125 μg MTb was administered. Mice were injected with Bordetella pertussis toxin (Sigma) i.p.: 1 μg per mouse for B10.RIII mice and 1.5 μg per mouse for C57BL/6 mice. To generate bone marrow chimeras, mice were lethally irradiated with 1000 rad and rested for 24 h before bone marrow recon-stitution. Bone marrow cells were prepared from the tibias and femurs of irradiated C57BL/6 mice. To establish bone marrow chimeras, mice were lethally irradiated with 1000 rad and rested for 24 h before bone marrow recon-stitution. Bone marrow cells were prepared from the tibias and femurs of irradiated mice for 10 days followed by a single injection of bone marrow cells. Bone marrow cells were prepared from the tibias and femurs of irradiated mice that were lethally irradiated with 1000 rad and rested for 24 h before bone marrow recon-

**Cell preparation**

Splenocytes were prepared by disrupting spleens to form a single cell sus-

**Flow cytometry and cell sorting**

Cell suspensions were incubated with 24G2 cell supernatant for 5 min at room temperature to block Fc receptors, primary mAb were added at op- timal concentrations as determined by titration, and cells were incubated at 4°C for 20 min. Intracellular staining was conducted using a cytokine/cyto-

**Cell culture and assessment of cell function**

Cells were cultured in round-bottom 96-well plates in complete medium; except where stated, 1 × 10^5 cells were added per well. For polyclonal proliferation, cells were stimulated with a whole retinal infiltrate, we did not detect the proliferation of retinal T cells at any time (data not shown).

**Results**

EAU induction in B10.RIII mice by active immunization with RBP-3(161–180) results in the influx of leukocytes into the eye, which reaches a peak 13 days later (10). After this time point, leukocyte cell numbers drop rapidly and further T cell expansion is not ob-

**Statistical analyses**

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

**Discussion**

The infiltration of CD4^+ T cells into the eye, which reaches a peak 13 days later (10). After this time point, leukocyte cell numbers drop rapidly and further T cell expansion is not ob-

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from retinal digests by adherence to plastic. The resulting nonadherent cells and adherent cells were stimulated separately with anti-CD3/anti-CD28 mAbs. The adherent cell population did not proliferate, while the nonadherent cells, which were depleted of macrophages, did proliferate in response to polyclonal T cell re-stimulation (supplementary Fig. 1). To examine whether or not retinal-infiltrating T cells were able to proliferate in the absence of macrophages, we also sorted CD4\(^{+}\)/H11001 and CD4\(^{+}\)/H11002 cells from retinal digests using magnetic beads. Activation of these populations independently indicated that purified CD4\(^{+}\)/H11001 T cells from the retinas of mice with EAU were able to proliferate in response to stimulation when separated from infiltrating myeloid cells (Fig. 2A). To demonstrate that this separation protocol did indeed remove macrophages from CD4\(^{+}\) fractions, we also restimulated cells with LPS and measured the production of NO. LPS stimulation of whole retinal digests led to high levels of NO produced by macrophages (Fig. 2B), similar to the level of NO that was produced by LPS-stimulated CD4\(^{+}\) retinal cells (data not shown), while no NO was elaborated by LPS-stimulated CD4\(^{+}\) cells (Fig. 2B). This demonstrates that purification successfully removed macrophages from the CD4\(^{+}\) T cells.

The interaction of T cells with macrophages leads to the production of IL-6 (Fig. 1D) and NO (Fig. 2B), and as well IFN-\(\gamma\) from the T cells (Fig. 1D). In this microenvironment, the T cells do not proliferate. In mixed leukocyte populations, macrophage activation can result from stimulation by T cell-produced IFN-\(\gamma\), leading to secretion of IL-6 and NO. To test whether or not macrophage activation by IFN-\(\gamma\) is the key to the control of T cell proliferation
by myeloid cells, we examined the effect of neutralizing this cytokine. Stimulating with anti-CD3/anti-CD28 and blocking IFN-γ led to an 80% reduction in NO production by retinal-infiltrating cells, and preventing macrophage activation in this manner also permitted T cell proliferation (Fig. 2, C and D).

Having established that the mutual activation of retinal-infiltrating CD4⁺ T cells and CD11b⁺ retinal-infiltrating myeloid cells suppressed T cell proliferation, we then addressed how potent these cells were, compared with APCs obtained from the spleen. We purified splenic CD4⁺ T cells and added either CD4-depleted leukocytes isolated from the retinas of B10.RIII mice at the peak of EAU or CD4-depleted leukocytes isolated from the spleen. The addition of CD4⁺ eye cells inhibited the proliferation of CD4⁺ T cells stimulated by anti-CD3/CD28 (Fig. 3A). In contrast, the splenic CD4⁺ cells did not reduce proliferation, but instead led to enhanced proliferation.

CD4-depleted spleen cells comprise a mixture of different APCs, including a large percentage of B cells. To compare the inhibitory ability of two equivalent myeloid populations more precisely, we prepared highly purified populations of CD45⁺CD11b⁺ cells by flow cytometric cell sorting from retinal isolates and from splenocytes. We added increasing numbers of these cells to cultures of whole splenocytes from mice immunized for EAU and restimulated with RBP-3₁₆₁–₁₈₀ peptide. CD11b⁺ cells obtained from the eye were able to inhibit T cell proliferation by 50% or more when added at a ratio of 1:100 or greater (Fig. 3B).

To date, many reports have established the potent ability of myeloid cells that infiltrate tumors to inhibit T cell activation. One hallmark of this inhibition is the down-regulation of T cell-expressed CD3 (22, 23). To determine whether or not CD4⁺ T cells prepared from the inflamed retina have such a phenotype, we compared the CD3 expression of CD4⁺ splenocytes with that of CD4⁺ cells obtained from the retina and found that it was reduced (Fig. 3D). The contribution of T cell activation to this phenotype is unclear from these experiments and this may play a role in preventing proliferation.

These experiments have demonstrated that myeloid cells from retinas of mice with EAU limited the proliferation, but not the cytokine production, of CD4⁺ T cells. Furthermore, such regulation required T cell-produced IFN-γ and could be reversed by IFN-γ neutralization. In EAU, IFN-γ acts on macrophages to induce the autocrine TNF-α that is necessary for NO production (14). Therefore, to investigate the importance of TNF-α signaling in the inhibition of T cell proliferation, we compared the retinal infiltrates in C57BL/6 WT and TNFR1 knockout mice at peak EAU. In addition, these assays would also allow us to confirm whether or not myeloid cell regulation of T cell proliferation also occurred in a second mouse strain in which EAU was induced with...
a different RBP-3 epitope. The characteristics of EAU induced in mice on a C57BL/6 background differ to those of the disease induced in B10.RIII mice, with a later peak of leukocyte infiltration into the retina and a later onset of clinical disease. However, the retinal infiltrate itself has a similar makeup to that described in B10.RIII animals, although the proportion of CD4\(^+\)/H11001 cells is somewhat higher (Fig 4A).

We characterized the responses of retinal infiltrate from WT and TNFR1/H11002/H11002 mice by sorting CD45\(^+\)CD11b\(^+\)CD4\(^+\)/H11001 cells from WT or TNFR1/H11002/H11002 retinas and restimulating them with anti-CD3/CD28 in the presence or absence of CD45\(^+\)CD11b\(^+\)CD4\(^+\)/H11002 cells sorted from the same retinas. CD11b\(^+\) cells from WT mice, but not TNFR1\(^{-/-}\) mice, inhibited T cell proliferation (Fig. 4A). Next, we examined the retinal-infiltrating CD4\(^+\) cells from TNFR1\(^{-/-}\) mice for hallmarks of regulation by myeloid cells. CD11b\(^+\) cells from WT mice expressed lower levels of CD31, a marker that has been associated with myeloid cells that inhibit T cell proliferation (24), compared with myeloid cells infiltrating the retinas of TNFR1\(^{-/-}\)/H11002/H11002 mice (Fig. 4C). Additionally, fewer infiltrating CD4\(^+\)/H11001 T cells from TNFR1\(^{-/-}\) mice had down-regulated CD3\(^\alpha\) compared with those from immunized WT mice (Fig. 4D). To compare the inhibitory potential of macrophages from WT and TNFR1\(^{-/-}\) animals directly, we examined the effect of CD11b\(^+\) leukocytes from TNFR1\(^{-/-}\) mice on the proliferation of WT splenic T cells. Purified CD11b\(^+\) cells, taken from the retina at the peak of disease, inhibited Ag-specific splenic T cell proliferation if these cells came from WT but not if they came from TNFR1\(^{-/-}\) mice (Fig. 4E).
Because TNFR1 expression appeared to influence the ability of macrophages to regulate T cell expansion ex vivo, we assessed retinal infiltrates in WT and TNFR1<sup>−/−</sup> mice over the EAU disease course for any evidence that this was also true in vivo. Fewer cells infiltrated the retinas of TNFR1<sup>−/−</sup> mice than the retinas of WT mice (Fig. 4F). However, the relative difference in leukocyte numbers between the two genotypes reached a peak around day 24. Strikingly, although CD11b<sup>+</sup> cells greatly outnumbered CD4<sup>+</sup> cells in early disease in both mouse strains, at subsequent time points the retinal infiltrate in TNFR1<sup>−/−</sup> mice was dominated by CD4<sup>+</sup> T cells (Fig. 4G). As previously reported (10), in WT mice, the ratio of CD4:CD11b<sup>+</sup> cells in the retina approximates to 1:1 only during the later resolution phase of EAU. Thus, during the course of EAU, WT myeloid cells that infiltrate the retina are able to regulate T cell numbers, while in mice that lack TNFR1 signaling this regulation is impaired. T cell numbers increase in...
relation to myeloid cells in TNFR1−/− mice, whereas as in WT mice, T cell numbers are restrained and myeloid cells predominate.

The reduction in NO from TNFR1−/− macrophages provides one explanation for the control of T cell proliferation in WT mice and the lack of tissue damage seen when EAU is induced in TNFR1−/− animals. However, it seems unlikely that this also explains the reduction in infiltration, because we have now shown that TNFR1−/− cells provoke more T cell proliferation that their WT counterparts and that T cells expand more in the retinas of TNFR1−/− mice. To shed further light on this, we addressed whether or not the lack of TNFR1 would have an impact on other aspects of the development of uveitis by constructing mixed bone marrow chimeras. We reconstituted lethally irradiated animals with TNFR1−/− and WT bone marrow cells at a ratio of 1:1 using WT cells that expressed a congenic form of CD45 (Ly5.2), and TNFR1−/− cells expressing Ly5.1. These (1:1-TNFR1−/−+Ly5.2 → Ly5.2) mice were immunized with RBP-31–20 peptide to induce EAU. Control chimeric mice that received either TNFR1−/−, (TNFR1−/− → Ly5.2), or WT, (WT → Ly5.2), bone marrow cells were similarly immunized to allow for any effect of chimera generation on EAU induction.

As expected, animals reconstituted with WT bone marrow developed EAU and mice reconstituted with TNFR1−/− bone marrow were resistant to EAU (Fig. 5A). The mixed chimeras, reconstituted with cells of both WT and TNFR1−/− genotypes had increased levels of ocular infiltration as compared with WT mice. In these mice, Ly5.1+ TNFR1−/− cells formed a significant proportion of the CD4+ cell population infiltrating the eye. In contrast, only a very few Ly5.1+ TNFR1−/− CD11b+ cells had entered the retina, where over 95% of the infiltrating CD11b+ cells originated from Ly5.2+ WT cells. The few CD11b+Ly5.1+ TNFR1−/− that did infiltrate the retinas of the mixed chimeras expressed much lower levels of CD31 and intracellular iNOS (18), than the CD11b+Ly5.2+ WT cells. These data indicate that TNFR1−/− CD4+ and WT CD4+ T cells have very similar trafficking properties during EAU while CD11b+ T cells that do not express TNFR1 are severely limited in their ability to access the target organ. Therefore, the lack of myeloid-dependent suppression in the TNFR1−/− mice is compensated for by a concomitant failure of these APCs to traffic to the eye, acquire Ag, and drive the autoimmune process forward.

**Discussion**

In this study, we have demonstrated that the microenvironment in the target organ during ocular autoimmunity is significantly different to that in the secondary lymphoid compartment. In the spleen and the lymph nodes, autoantigen-specific T cell responses are amplified by T cell proliferation. This generates both the effecter T cells, which migrate into target organs and coordinate inflammation, and the T cell populations, which are destined to become part of the memory pool that provides enhanced immune surveillance. In contrast, once Ag-specific CD4+ T cells have migrated into the retina during ocular autoimmunity, they respond to stimulation by secreting proinflammatory cytokines, but their proliferation is profoundly restricted. This restriction depends on CD11b+ myeloid regulatory cells, which elicit cytokine production, but curtail T cell proliferation in a TNFR1-dependent fashion. T cells that escape this environment have the potential to resume proliferation.

The role of myeloid cells in promoting tissue damage in target organs during autoimmunity has been carefully elucidated (25–27). However, there is increasing evidence of another aspect to myeloid cell function (19): the ability of populations of immature macrophage-like cells to suppress Ag-specific T cell proliferation (28). For example APCs, activated in the presence of TGF-β, a cytokine present at high concentration in the eye, have been reported that generate regulatory T cells rather than inflammatory responses (29).
In tumors, a heterogeneous population of myeloid cells that restrict immune responses has been clearly identified (30). These cells are called myeloid-derived suppressor cells (MDSCs), and their presence correlates with a poor prognosis (31). MDSCs express CD11b and Gr-1, as well as the macrophage marker F4/80, and suppress antitumor T cell proliferation (32–35). It has been argued that these cells prevent effective immunosurveillance of tumors (24, 36, 37), but it is difficult to rationalize this as a useful physiological function. We believe that a more complete explanation for the accumulation of inhibitory myeloid cells as inflammation progresses is required. We speculate that this accumulation may serve to limit tissue damage, conserve scarce nutrients, or both. One mechanism by which myeloid cells affect T cell proliferation is via arginine metabolism (38). Local depletion of arginine results in down-regulation of CD3ζ (39), dampening T cells responses. As we observe similar CD3ζ down-regulation among retinal T cells, this mechanism may also play a role in regulation of T cell responses in the target organ during autoimmunity, but it is likely that other mechanisms may also be involved. We propose that myeloid-dependent inhibition of proliferation may be a common finding in organ-specific autoimmune disease, and in support of this is the description of the regulation of T cell proliferation by splenic myeloid cells during EAE (40).

These myeloid cells do not merely inhibit T cell activation. Instead, they appear to divert T cell activity, suppressing proliferative function and the production of some soluble factors, while at the same time permitting the release of high levels of proinflammatory cytokines. Such activity is more akin to a local regulation of immune responses by populations of regulatory T cells, rather than a pan-immunosuppression within the target organ. Critical, it appears that the ability of these myeloid regulatory cells to dampen T cell proliferation is dependant upon activation of the myeloid cells via TNFR1. Thus, this process may be reversible by therapies that target the TNFR1 pathway. Indeed, blockade of TNFR1 in rats with EAU leads to suppression of myeloid cell function (41). Thus, TNFR1 expression by macrophages is necessary for the inhibitory function of these cells and this may also be relevant to the development of MDSCs that promote tumor progression. This suggestion is supported by the report that TNFR1⁻/⁻ mice are resistant to liver tumor formation (42).

In addition to its important effect on the generation of regulatory myeloid cells, we have also demonstrated that TNFR1 signaling plays a nonredundant role in the control of infiltration by myeloid cells, but not T cells, into target organs. Indeed, this mechanism provides an alternative explanation for the differences observed between retinal infiltrates during EAU progression in TNFR1⁻/⁻ mice and WT mice. Previously, we suggested that the greater proportion of T cells observed in TNFR1⁻/⁻ retinas may be due to the absence of regulatory myeloid cells, which, in WT retinas, prevent T cell proliferation. However, it is more likely that this situation reflects the deficit of TNFR1⁻/⁻ myeloid cells to migrate into the target organ. In EAU studies in rats, there was some indication that neutralizing TNF-α activity led to an alternation in the populations of immune cells observed in the retina, with increased T cell numbers as compared with macrophages (43). EAE studies in TNF-α knockout mice also indicate a role for TNF-α in reducing the relative number of myeloid cells infiltrating the target organ: there is an increase in the relative proportion of CD4⁺CD45⁻CD4⁻ cells in the CNS of WT mice with EAE as compared with TNF-α knockout mice (44). These data support our finding that a lack of TNF-α signaling can lead to a relative increase in infiltration of CD4⁺ T cells into the target organ.

Earlier studies have concluded that autocrine TNF-α signaling controls macrophage effector function in tissues (14, 41, 45, 46). However, the critical and selective part that TNFR1 signaling plays in macrophage localization, which we have demonstrated herein, may have an essential role in the early stages of uveitis, when leukocytes move rapidly into the target organ (10). This also raises the possibility that targeting TNFR1 signaling might preserve immune surveillance by T cells while inhibiting the damaging inflammation driven by macrophages.

This study highlights the essential role that TNFR1 plays in the determining the function of activated myeloid cells. Not only does TNFR1 signaling appear to control macrophage migration in target organs during autoimmunity, but once in the target organ such signals can generate potent regulation of T cell activity. These processes may be critical in determining the outcome of autoimmune responses as well as anti-tumor responses. As such, this pathway is an attractive target in the treatment of organ-specific autoimmune disease and the modulation of antitumor responses.

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Disclosures
The authors have no financial conflict of interest.

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