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Functional Macrophage Heterogeneity in a Mouse Model of Autoimmune Central Nervous System Pathology

Anat London,*-† Inbal Benhar,*-† Mary J. Mattapallil, † Matthias Mack, ‡ Rachel R. Caspi, † and Michal Schwartz*

Functional macrophage heterogeneity is well appreciated outside the CNS in wound healing and cancer, and was recently also demonstrated in several CNS compartments after “sterile” insults. Yet, such heterogeneity was largely overlooked in the context of inflammatory autoimmune pathology, in which macrophages were mainly associated with disease induction and propagation. In this article, we show the diversity of monocyte-derived macrophages along the course of experimental autoimmune uveitis, an inflammatory condition affecting the ocular system, serving as a model for CNS autoimmune pathology. Disease induction resulted in the appearance of a distinct myeloid population in the retina, and in the infiltration of monocyte-derived macrophages that were absent from control eyes. During the disease course, the frequency of CX3CR1high infiltrating macrophages that express markers associated with inflammation-resolving activity was increased, along with a decrease in the frequency of inflammation-associated Ly6C+ macrophages. Inhibition of monocyte infiltration at the induction phase of experimental autoimmune uveitis prevented disease onset, whereas monocyte depletion at the resolution phase resulted in a decrease in Foxp3+ regulatory T cells and in exacerbated disease. Thus, monocyte-derived macrophages display distinct phenotypes throughout the disease course, even in an immune-induced pathology, reflecting their differential roles in disease induction and resolution. The Journal of Immunology, 2013, 190: 3570–3578.

Macrophages are key players in settings of sterile insults, as well as under infectious conditions and in cancer. Outside the CNS, the heterogeneity and multifunctionality of these cells is well substantiated (1–4). Monocyte-derived macrophages, which infiltrate the tissue upon insult, have generally been divided into two subsets: CCR2+CX3CR1lowLy6C+ monocyte-derived macrophages are the first recruited in response to insult and exhibit a typical proinflammatory phenotype, whereas the second subset, composed of CCR2+CX3CR1highLy6C- cells, takes part in immune resolution (2, 5–7). Macrophages are also broadly divided, based on their activation state, into two polarized phenotypes known as M1, or classically activated macrophages, and M2, or alternatively activated macrophages (3), which include “resolution-phase macrophages” (8). Recently, Mosser and Edwards (4) suggested classifying macrophages based on their functions in helping to maintain homeostasis, namely, host defense, wound healing, and immune regulation. All of these classifications indicate the diversity of macrophage populations, displaying functions that reflect the changing needs of the tissue along the course of healing (5, 7), including clearance of dead cells and cellular debris, as well as secretion of proinflammatory and anti-inflammatory molecules and growth factors. The timing and duration of the macrophage response, as well as their phenotype, which depends on the local milieu within the tissue and on their route of trafficking (4, 9), all together determine the fate of the tissue.

In the CNS, macrophage heterogeneity has been demonstrated under various sterile inflammatory conditions and in models of neurodegenerative disease (10–13). Those studies functionally distinguished between resident microglia, the native myeloid cells of the CNS, and blood-borne monocyte-derived macrophages that infiltrate the CNS only after an insult. Monocyte-derived macrophages have been shown to serve as resolution-phase macrophages, required to bring about the proper termination of the immune response and the restoration of homeostasis in the tissue, thereby promoting neuroprotection and neurorepair (10, 11, 14). In this study, we explored the possibility that distinct subsets of macrophages are differentially involved in coping with autoimmune CNS pathology, using the model of experimental autoimmune uveitis (EAU). EAU is induced in mice by immunization with retinal Ags and is a model for human posterior uveitis, a potentially blinding inflammatory ocular condition that affects the choroid of the eye and the neural retina (15, 16). Many studies have identified macrophages as key players in EAU, mainly in the context of the induction and effector phase of the disease (17, 18), although several reports have indicated the presence of macrophages in the resolution stage as well (19, 20). However, in most of these studies, there was no functional distinction between resident microglia and infiltrating macrophages. In addition, the current state of knowledge on macrophage involvement in EAU

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A.L. and I.B. designed and performed the research, collected, analyzed, and interpreted the data, performed statistical analysis, and wrote the manuscript. M.J.M. analyzed the rd8 status of the mice. M.M. contributed the MC-21 Ab. R.R.C. participated in designing the research and establishing the experimental autoimmune uveitis model, and contributed to writing the manuscript. M.S. designed the research, interpreted the data, and wrote the manuscript.

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Abbreviations used in this article: BM, bone marrow; EAU, experimental autoimmune uveitis; OCL, ganglion cell layer; IB-4, isolectin B4; RRG, retinoid-binding protein; RGC, retinal ganglion cell; Treg, regulatory T cell; WT, wild type.
reinforces the need for characterizing distinct subsets of monocyte-derived macrophages and their functions at different phases of the disease in vivo.

In this study, we followed the changes in myeloid populations along the course of EAU and identified infiltration of monocytic macrophages into diseased retinas. We found that these cells are composed of CX3CR1high and CX3CR1low macrophage populations, whose kinetics change along the disease course. The frequency of CX3CR1high macrophages was highest at the peak of disease and remained elevated throughout the resolution phase; these cells were found to express higher levels of resolution-associated surface markers compared with their CX3CR1low counterparts. Inhibition of monocyte-derived macrophage infiltration at disease onset prevented EAU, whereas inhibition of this infiltration after the peak of disease resulted in a decrease in Foxp3+ regulatory T cells (Tregs) in the retina, and in worse disease outcome. These results indicate that monocyte-derived macrophages are functionally heterogeneous in a CNS autoimmune disease and may differentially contribute to disease resolution as compared with disease induction. Thus, particular macrophage subsets can play a role in disease resolution, even in a pathology that is primarily induced by an aberrant immune response.

Materials and Methods

Mice

Adult male (8–10 wk old) C57BL/6J mice and heterozygous Cx3cr1GFP/+ transgenic mice (B6.129P-Cx3cr1<sup>GFP</sup>tm1Lui/J, in which one of the Cx3CR1 chemokine receptor alleles is replaced with a gene encoding GFP) (21) were used. These mice were tested and found negative for the rd8 mutation (22). Foxp3<sup>GFP</sup> mice were a generous gift from Dr. Alexander Rudensky. Animals were supplied by the Animal Breeding Center of the Weizmann Institute of Science. All experiments conformed to the regulations formulated by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

Preparation of bone marrow chimeras

To create [Cx3CR1<sup>GFP</sup>→ WT] bone marrow (BM) chimeric mice, wild type (WT) recipient mice received lethal whole-body irradiation (950 rad) while shielding the head, as previously described (11, 23). This shielding prevented any direct effects on the retina and/or infiltration of myeloid cells other than those related to disease induction. On the next day, mice were reconstituted with 5 × 10<sup>6</sup> Cx3CR1<sup>GFP</sup> BM cells according to a previously described protocol (11). This protocol results in BM chimerism rates of 50–70%. Chimeric mice were subjected to EAU induction 8–12 wk after BM transplantation.

EAU induction and scoring

Native bovine interphotoreceptor retinoid-binding protein (IRBP) was produced as described previously (24). The uveitogenic 20-aa peptide or with 300 μg native bovine IRBP as a 1:1 emulsion with CFA containing Mycobacterium tuberculosis strain H37RA at 2.5 mg/ml (1.25 mg/ml final). Mice were coinjected with 1 μg Bordetella pertussis toxin (Sigma) ip. In this model, which induced disease in 80–100% of the mice, clinical onset is described around day 15, and disease peak is between days 22 and 29 (26–28).

For EAU scoring, eyes were processed for histopathology on day 35 after immunization and stained with H&E. EAU severity was evaluated in a blinded fashion on a scale of 0–4, using previously published criteria (29, 30). The following Abs were used for immunolabeling: rabbit anti-GFP (1:100; MBL), mouse anti-Brd3a (1:50; Santa Cruz Biotechnology), goat anti-IL-10 (1:20; R&D Systems). The M.O.M. immunodetection kit (Vector Laboratories) was used to localize mouse primary Abs. For activated myeloid cell labeling, FITC-conjugated Bandeiraea simplicifolia isolectin B4 (IB-4; 1:50; Sigma-Aldrich) was added for 1 h to the secondary Ab solution. Secondary Abs included Cy2/Cy3-conjugated donkey antiserum, -rabbit, or -goat Abs (1:150–200; all from Jackson ImmunoResearch Laboratories). The slides were exposed to Hoechst stain (1:2000; Invitrogen) for 1 min. For microscopic analysis, a fluorescence microscope (Eclipse 80i; Nikon) was used. The fluorescence microscope was equipped with a digital camera (DXM1200F; Nikon) and with either a 20× NA 0.50 or 40× NA 0.75 objective lens (Plan Fluor; Nikon). Recordings were made on postfixed tissues at 24°C using NIS-Elements, F3 (Nikon) acquisition software. Images were cropped, merged, and optimized using Photoshop (Adobe), by making minor adjustments to contrast and were arranged using Canvas X (Deneba Software).

Isolation of retinal cells and flow cytometry

After intracardiac perfusion with PBS, retinas were removed by dissection and processed to single-cell suspension, as previously described (19, 33). The following fluorochrome-labeled Abs were purchased from BD, Biologend, eBioscience, or AbD Serotec, and were used according to the manufacturers’ protocols: PE-conjugated anti-CD11b, CD206 (MMR), and IL-4Ra Abs; PerCP-cy5.5–conjugated anti-Ly6C and CD11b Abs; allophycocyanin-conjugated anti-CD115, TCRβ, Foxp3, and CD204 Abs; Alexa 647–conjugated anti-Dectin-1 Ab; and Pacific Blue/Brilliant Violet–conjugated anti-TCRβ, CD4, and CD45.2 Abs. Foxp3 staining was performed using the Foxp3 staining buffer set (eBioscience), and IL-10 staining was performed using Mouse IL-10 Secretion Assay Detection Kit (Miltenyi Biotec), according to manufacturers’ protocols. Cells were analyzed on a FACS LSRII cytometer using FACSdiva software (both from BD). Analysis was performed with FlowJo software (Tree Star). In each experiment, relevant negative and positive control groups were used to determine the populations of interest and to exclude the rest.

Statistical analysis

Levene’s test was used to check equality of variance. In the case of equal variances, data were analyzed using a Student t test to compare between two groups, or by one-way ANOVA to compare several groups. Tukey’s HSD post hoc test was used for follow-up pairwise comparison of groups after the null hypothesis was rejected (p < 0.05). In the case of unequal variances, data were log-transformed to achieve equal variances when possible; otherwise, the Kruskal–Wallis test was used to compare several groups, followed by Dunn’s test. EAU scores were analyzed by Mann–Whitney U test. Results are presented as mean ± SE, and y-axis error bars in the graphs represent SE, unless indicated otherwise.

Results

Changes in myeloid populations after EAU induction

EAU was induced in mice by immunization with a uveitogenic regimen of IRBP 1–20, as described in Materials and Methods. This regimen induced disease in 80–100% of animals by 14 d after immunization. To track changes in mononuclear myeloid cells after induction of EAU, we first used mice that express GFP under the Cx3CR1 promoter (21). In these mice, the GFP label enables the entire mononuclear myeloid population to be followed, with no distinction between resident microglia and infiltrating monocytic-derived macrophages. Mice were left untreated (naive) or immunized with either IRBP/CFA or PBS/CFA. Immunohistochemical staining for GFP at time points corresponding to the peak of disease, which occurs between days 22 and 29 after immunization (25, 27, 28, 34), revealed numerous CX3CR1–GFP<sup>+</sup> myeloid cells dispersed among the retinal layers in the IRBP/CFA group. Some of these cells were localized to the photoreceptor layer and the ganglion cell layer (GCL; Fig. 1A). Costaining with the retinal ganglion cell (RGC) marker, Brn3a, revealed a distorted GCL,
Changes in myeloid populations after EAU induction. Retinas taken at the peak of EAU from Cx3cr1GFP/+ mice immunized with either PBS/CFA or IRBP/CFA were analyzed by immunohistochemistry and flow cytometry. (A) Staining for Brn3a (red), a specific marker for RGCs, and for GFP (green), representing CX3-CR1+ myeloid cells. Insets show higher magnification. Scale bars are depicted in micrometers. (B) Immunohistochemical analysis of IB-4+ cells (green), representing activated macrophages, located in the various retinal layers, indicated by Hoechst staining (blue), including the GCL. (C) Retinal leukocytes (CD45+), analyzed for CX3CR1-GFP and CD11b expression to identify myeloid cells, in Cx3cr1GFP/+ mice either left untreated (naive, i), or immunized with PBS/CFA (ii) or IRBP/CFA (iii). Note the appearance of the distinct CD11b+CX3-CR1-GFP+ subset in the IRBP/CFA vaccinated mice, alongside an increase in CD11b+CX3-CR1-GFPhigh myeloid cells. (D and E) Bar graphs show quantification of leukocytes and total myeloid cells, respectively, in retinas from the different groups. Graphs throughout the figure show mean ± SE of each group. *p < 0.05.

Monocyte-derived macrophages infiltrate uveitic retinas

To address the question of whether the changes detected in myeloid populations include recruitment of monocytes from the blood, we performed the EAU immunization protocol on Cx3cr1GFP/+→WT BM chimeric mice, whose WT BM was replaced with that of Cx3cr1GFP/+ mice, and followed the infiltration of monocyte-derived macrophages into the retina at several time points after EAU induction. In these chimeras, it is possible to distinguish between infiltrating monocyte-derived macrophages and resident microglia, as only the infiltrating cells, derived from donor BM, are labeled with GFP.

Retinas from chimeric mice that received PBS/CFA showed no infiltration of circulating monocytes at any time point after immunization, as seen by both flow cytometric and immunohistochemical analyses, and were identical to naive mice (Fig. 2A, left panels, 2B, 2C, top panel). In contrast, retinas of IRBP/CFA mice contained many infiltrating GFP+ monocyte-derived macrophages, which were scattered among the retinal layers and were found in a state of activation, as seen by IB-4 staining (Fig. 2A, right panel, 2B, 2C, bottom panel). We followed the kinetics of this infiltration along the disease course and found that the time of peak monocyte infiltration corresponded to the peak of disease in this model, namely, days 22–29 postimmunization (Fig. 2D). Of note, the CD11b+CX3-CR1-GFP+ subset, mostly representing the resident myeloid cells (and possibly neutrophils, to some extent), was also found to increase along the course of EAU (from 237.21 ± 127.96 cells at EAU onset [day 15] to 782.61 ± 235.73 at disease peak [day 22]; p < 0.001).

Interestingly, some of the infiltrating macrophages were found to express the anti-inflammatory cytokine, IL-10, as was demonstrated both by flow cytometry and immunohistochemical staining starting from the peak of EAU (Fig. 2E, 2F). Because the immune infiltrate in uveitis is usually considered a measure of inflammation and disease severity, detecting the expression of this classical anti-inflammatory cytokine by some of the infiltrating macrophages suggests that the macrophage infiltrate in this disease is rather heterogeneous.

Monocyte-derived macrophage heterogeneity over the course of EAU

To address macrophage heterogeneity in EAU, we induced the disease in Cx3cr1GFP/+→WT BM chimeric mice and evaluated...
the phenotype of the infiltrating macrophages in the retina using flow cytometry. Several studies that characterized subgroups of monocyte-derived macrophages in insults outside the CNS divided these cells into two main subsets based on the expression of two markers: the CX3CR1lowLy6C+ and CX3CR1highLy6C2 populations, corresponding to proinflammatory and anti-inflammatory phenotypes, respectively (5, 7). Gating on the infiltrating monocyte-derived macrophages at the peak of EAU, we found that these cells could be subdivided based on the intensity of CX3CR1-GFP expression (Fig. 3A). The frequency of CX3CR1-GFPhigh macrophages was low at the onset of disease, after which it began to increase, reaching peak levels at day 22, the peak of disease, which, in effect, marks the beginning of resolution; this population remained high throughout the resolution phase (Fig. 3B). In line with this increase, the frequency of Ly6C+ macrophages, corresponding to the proinflammatory subset, was highest at disease onset and decreased by day 22, remaining relatively low at the later stages of EAU (Fig. 3C). We found that the initial appearance

**FIGURE 2.** Monocyte-derived macrophages infiltrate diseased retinas in EAU. [Cx3cr1GFP+/→ WT] BM chimeric mice were subjected to the EAU immunization protocol, and their retinas were analyzed for the presence of infiltrating monocyte-derived macrophages at the peak of disease (A–C). (A and B) Representative dot plots and their quantitative analysis, showing infiltration of CD11b⁺CX3CR1-GFP⁺ monocyte-derived macrophages only to retinas from mice vaccinated with IRBP/CFA, and not to retinas from naive or PBS/CFA-vaccinated mice. (C) Immunohistochemical analysis of retinal sections for activated infiltrating monocyte-derived macrophages. IB-4, green; CX3.CR1-GFP, red; Hoechst, blue. Arrows indicate double-labeled cells. (D) Quantitative analysis showing the kinetics of monocyte-derived macrophage infiltration along the disease course, as analyzed by flow cytometry. (E and F) IL-10 expression by monocyte-derived macrophages in EAU, as analyzed by flow cytometry (IL-10⁺, black curve; isotype control, gray curve) and by immunohistochemical analysis (IL-10, red; CX3.CR1-GFP, green), respectively. Scale bars are depicted in micrometers. Graphs throughout the figure show mean ± SE of each group. *p < 0.05, **p < 0.01.
Monocyte-derived macrophages in EAU are a heterogeneous population. (A) CX3CR1-GFP\(^+\) mean fluorescence intensity of infiltrating monocyte-derived macrophages from the peak of EAU, showing that they can be divided into two subsets, CX3CR1-GFP\(^{low}\) (blue curve) and CX3CR1-GFP\(^{high}\) (red curve). (B) Quantitative analysis showing the kinetics of the frequency of CX3CR1-GFP\(^{high}\) cells out of total infiltrating macrophages along the disease course. (C) Quantitative analysis showing the kinetics of the frequency of Ly6C\(^+\) cells out of total infiltrating macrophages along the disease course. (D) Representative histograms showing expression levels of various markers by the CX3CR1-GFP\(^{low}\) and CX3CR1-GFP\(^{high}\) infiltrating macrophages, as well as the CX3CR1-GFP\(^{-}\) myeloid cells in the retina, at the peak of EAU. Bars demarcate cells positive for the indicated marker. Numbers above bars represent percentage of cells positive for the indicated marker out of the relevant population; CX3CR1-GFP\(^{low}\), blue; CX3CR1-GFP\(^{high}\), red; CX3CR1-GFP\(^{-}\), green; isotype control, gray. Graphs throughout the figure show mean ± SE of each group. \(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\). ns, Nonsignificant.

of the CX3CR1-GFP\(^{high}\) macrophages corresponded to the timing of the beginning of the resolution phase. This observation, together with the well-recognized contribution of the CX3CR1\(^{high}\) population to wound healing and repair under injurious conditions in non-CNS tissues, prompted us to examine the possibility that this subset might also take part in the resolution of EAU. We therefore tested these cells for expression of markers known to be associated with immune resolution and wound healing, namely, CD206 (mannose receptor), Dectin-1, CD204 (macrophage scavenger receptor 1), and IL-4R\(\alpha\). At all time points tested, these markers were found to be expressed at higher levels among the CX3CR1-GFP\(^{high}\) population, as compared with the CX3CR1-GFP\(^{low}\) cells (Fig. 3D). Notably, the CX3CR1-GFP\(^{high}\) population was found to express lower levels of Ly6C relative to its CX3CR1-GFP\(^{low}\) counterpart, reminiscent of the anti-inflammatory monocyte subset described by others (5, 7).

In CNS insult, another level of complexity is introduced when taking into account the microglia, the resident immune population of the CNS, which have a low rate of turnover by cell renewal, independently of circulating monocytes (39–41), and actively participate in the local inflammatory response. To gain insight regarding the phenotype of the resident myeloid cells, we gated on the CX3CR1-GFP\(^{-}\) population in retinas from (Cx3cr1\(^{GFP}\) \(\rightarrow\) WT) BM chimeric mice. Interestingly, the frequencies at which the resolution markers were expressed within this population were significantly lower when compared with the CX3CR1-GFP\(^{high}\) infiltrating macrophages, and comparable with those of the CX3CR1-GFP\(^{-}\) macrophages. However, the resident cells expressed Ly6C at a lower frequency than the CX3CR1-GFP\(^{low}\) macrophages (Fig. 3D). Together, these results demonstrate the diverse phenotypes displayed by macrophages in EAU, and suggest that this heterogeneity may also be reflected in terms of their function, which may affect disease outcome.

Early recruitment of monocyte-derived macrophages is required to induce EAU

In view of the significant phenotypic differences we found between the two subsets of infiltrating monocyte-derived macrophages, we next turned to explore the roles of these cells at different phases of EAU. Because the CX3CR1-GFP\(^{high}\) macrophages, which exhibited a resolving phenotype, appeared at the beginning of the resolution phase, we hypothesized that inhibiting macrophage infiltration before the peak of EAU, as compared with after the peak, would result in different disease outcomes.

By making use of the anti-CCR2 Ab, MC-21, which depletes Ly6C\(^{+}\) monocytes from the blood (31), we were able to greatly reduce their infiltration into the retinas of IRBP/CFA-immunized mice (Fig. 4A–C). Such depletion starting before the onset of EAU resulted in diminished T cell infiltration into the retina as analyzed at day 22 after immunization, so that these retinas retained an appearance comparable with those of PBS/CFA-immunized (control) mice (Fig. 4D, 4E). Histological examination revealed that, as opposed to retinas from untreated IRBP/CFA-immunized mice that developed full-blown disease, retinas from MC-21–treated mice did not display the typical histopathological features of EAU, namely, vasculitis, retinal folding, and distortion of the retinal layers (29) (Fig. 4F). These results indicated that monocyte-derived macrophages recruited early in the disease process are involved in the induction of EAU pathology.

Monocyte-derived macrophages at the later phase of EAU contribute to disease resolution

To determine whether monocyte-derived macrophages are also involved in the resolution phase of EAU, we used the MC-21 Ab for the depletion of monocyte-derived macrophages starting at the peak of disease (Fig. 5A), a time when the CX3CR1-GFP\(^{high}\) population, reminiscent of resolution-phase macrophages, was at its highest (Fig. 3B). This depletion regimen resulted in lower numbers of CX3CR1-GFP\(^{+}\) infiltrating macrophages in the retina (Fig. 5B). Levels of Foxp3\(^{+}\) Tregs, whose elevation is characteristic of an inflammation-resolving milieu, and which have been associated with resolution of EAU (19, 42–45, and P.B. Silver, R. Horai, J. Chen, A.M. Hansen, W.C. McManigle, R. Villasmil, M.R. Kesen, and R.R. Caspi, manuscript in preparation), served as an initial criterion of resolution. Flow cytometric analysis revealed a significant increase in the number of CD4\(^{+}\)Foxp3\(^{+}\) Tregs in the
retina along the disease course (Fig. 5C), representing a 2.5-fold increase in frequency. Monocyte depletion affected the number of Tregs in the retina, as indicated by a significant decrease in the frequency of CD4\(^+\)Foxp3\(^+\) Tregs on day 35 after immunization, compared with retinas of IRBP-immunized mice after MC-21 treatment. (D and E) Representative dot plots and their quantitative analysis, showing the number of T cells in IRBP-immunized mice with and without MC-21 treatment, as compared with PBS-immunized controls. Asterisks above bars indicate significant differences compared with PBS/CFA controls. Significant differences between the IRBP and IRBP + MC-21 groups are indicated by asterisks between bars. (F) Representative H&E staining showing the histopathology of IRBP-immunized mice with and without MC-21 treatment. Note the typical features of EAU (massive immune infiltrate, retinal folds, vasculitis, and damage to the retinal layers) in the IRBP group, all of which are absent from the MC-21–treated group. Scale bars are depicted in micrometers. Graphs throughout the figure show mean \(\pm\) SE of each group. *\(p < 0.05\), ***\(p < 0.001\).

Discussion

The heterogeneity of myeloid-derived cells is a well-known phenomenon in cancer (46–48), wound healing (5, 7), and was recently also described in sterile CNS trauma (10, 11). In this study, such heterogeneity was demonstrated in an autoimmune pathological condition within the CNS, autoimmune uveitis. This diversity was manifested in this study by the presence of resident microglia, as well as infiltrating monocyte-derived macrophages in the uveitic retina, the latter being recruited only upon disease induction. Moreover, within the infiltrating macrophage population, we identified different phenotypic subsets, the frequencies of which changed along the disease course, and which appeared to have distinct functional effects, contributing differentially to disease induction and resolution. The fact that specific depletion of the infiltrating macrophages, when performed at the resolution phase, resulted in impaired EAU resolution indicates that monocyte-derived macrophages performed a role that could not be provided by the resident cells.
Macrophage plasticity was previously demonstrated in experimental autoimmune encephalomyelitis by an in vitro functional assay in which myeloid cells isolated from the CNS at different stages of the disease were capable of either activation or suppression of T cells (49). These results are consistent with our in vivo depletion experiments in which the absence of monocyte-derived macrophages before disease onset resulted in diminished T cell recruitment and prevented disease development, whereas monocyte-derived macrophage depletion at the later stage appeared to interfere with disease resolution. Another recent study in the experimental autoimmune encephalomyelitis model showed that invariant NKT cell activation results in the differentiation of monocytes into an M2 phenotype and has a positive impact on disease outcome (38). Thus, the plasticity of macrophages enables them to perform distinct and even opposing functions, which are probably influenced by the changing environmental cues within the tissue, reflecting its needs along the natural course of the disease. Whether the different macrophage subsets, as identified in this study, suggest their involvement in disease resolution and might account for the finding of Kerr et al., namely that myeloid cells from uveitic eyes can directly inhibit T cell proliferation in vitro (19). Interestingly, the infiltrating macrophages in our study were also found to express the classical anti-inflammatory mediator, IL-10, which has been shown to have a protective role in EAU (52) and is an important factor in the development and function of Tregs (53–56).

It is known that macrophages can promote immune suppression by recruiting Tregs or promoting their expansion in tumor tissues, contributing to tumor escape mechanisms (54, 57). Tregs have been associated with all stages of EAU and appear to be important in clinical uveitis. Several roles have been attributed to these cells: 1) they set the threshold of EAU susceptibility (43); 2) they accumulate in the eye during active disease (19, 42, 45); 3) they are involved in inducing and maintaining remission (P.B. Silver, R. Horai, J. Chen, A.M. Hansen, W.C. McManigle, R. Villasmi, M.R. Kesen, and R.R. Caspi, manuscript in preparation); and finally, 4) in clinical uveitis, decreased Treg number or function has been associated with disease (33, 44). Our results, showing that monocyte-derived macrophages affect the levels of Tregs in the eye, may indicate that this is one of the mechanisms by which specific macrophage subsets may be involved in disease resolution. In addition, it has been shown that an inflammatory environment dampens the suppressive capacity of Foxp3+ Tregs (58) and impairs the conversion of T cells into Tregs (59), it is conceivable that by controlling the inflammatory environment in the eye, the resolution-associated infiltrating macrophages character-

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Monocyte-derived macrophages contribute to EAU resolution. Mice were immunized for EAU and were treated, or not, with MC-21 after the peak of disease, as illustrated in the diagram (A). (B) Representative dot plots showing the decrease in the infiltration of monocyte-derived macrophages to retinas of IRBP-immunized mice after late MC-21 treatment. (C) Quantitative analysis showing an increase in CD4+Foxp3+ Tregs after the peak of EAU. (D) Quantitative analysis showing a decrease in the frequency of Foxp3+ Tregs after monocyte depletion. Graphs in (C) and (D) show mean ± SE of each group; *p < 0.05, **p < 0.01. (E) Representative H&E staining showing the histopathology of IRBP-immunized mice with and without MC-21 treatment at the resolution phase of EAU. Note the extensive damage to the retinal layers in the MC-21-treated group. (F) EAU scores of IRBP-immunized mice with and without MC-21 treatment at the resolution phase of EAU. Graph shows median and interquartile range of each group (p = 0.077).
ized in our study enable the Tregs to perform their regulatory functions at the resolution phase. Regardless of whether they might act directly or through other cells, our results indicate that even in a pathology such as EAU, which is immune induced, certain macrophage subtypes have an essential role in restoring immune homeostasis; these results are consistent with our previous studies in a model of noninflammatory insult to the eye induced by glutamate toxicity (10).

Several autoimmune diseases in humans, including posterior uveitis, show a relapsing-remitting pattern in which patients exhibit fluctuations in inflammatory activity as part of the natural course of the disease. In EAU, which serves as an animal model of posterior uveitis, disease typically reaches a phase of resolution, characterized by scarring and gliosis of the retina and decreased inflammatory infiltrate. However, in the C57BL/6 mouse strain, used in our study, the retina does not return to normal in terms of leukocyte levels; there are fluctuations in the numbers of both macrophages and T cells at the later phases of disease (19, 60). It has been suggested that this resolution pattern is facilitated by regulatory populations, which keep the disease in check by limiting the inflammatory infiltrate, thereby preventing disease relapse (19). Our results imply that the CX3CR1(high) infiltrating macrophages take part in this feedback mechanism, enabling the disease to reach a state of equilibrium, rather than to relapse. It is conceivable that the relapsing-remitting nature of autoimmune diseases in patients can be partly attributed to the insufficient recruitment/activation of the corresponding resolving macrophage population in humans, or its inadequate conversion into the required phenotype.

Immunosuppressive drugs are often prescribed to patients suffering from autoimmune disease. Such treatment might help relieve some symptoms of the disease at the early stage, presumably at disease onset, as is supported by our present findings. However, our results identifying a subset of macrophages with immune-resolving activity suggest the possibility that the disadvantage of immunosuppressive treatment could be interference with the recruitment of such cells at the advanced stages of the disease, where they appear to be essential for its resolution. Thus, the functional heterogeneity of macrophages in an autoimmune disease as demonstrated in this article, argues against the indiscriminate use of immunosuppressive drugs that might also interfere with those immune processes that resolve uncontrolled inflammation and are pivotal to healing. Our data thus argue in favor of therapeutic approaches aimed at inhibition or augmentation of specific immune populations at the right time, rather than at general immune suppression.

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Disclosures
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