The macrophage F4/80 receptor is required for the induction of antigen-specific efferent regulatory T cells in peripheral tolerance

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We show that the mouse macrophage-restricted F4/80 protein is not required for the development and distribution of tissue macrophages but is involved in the generation of antigen-specific efferent regulatory T (T reg) cells that suppress antigen-specific immunity. In the in vivo anterior chamber (a.c.)–associated immune deviation (ACAID) model of peripheral tolerance, a.c. inoculation of antigen into F4/80[−/−] mice was unable to induce efferent T reg cells and suppress delayed-type hypersensitivity (DTH) responses. Moreover, the use of anti-F4/80 mAb and F4/80[−/−] APCs in an in vitro ACAID model showed that all APC cells in the culture must be able to express F4/80 protein if efferent T reg cells were to be generated. In a low-dose oral tolerance model, WT but not F4/80[−/−] mice generated an efferent CD8^{+} T reg cell population that suppressed an antigen-specific DTH response. Peripheral tolerance was restored in F4/80[−/−] mice by adoptive transfer of F4/80^{+} APCs in both peripheral tolerance models, indicating a central role for the F4/80 molecule in the generation of efferent CD8^{+} T reg cells.

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Abbreviations used: Ab, antibody; a.c., anterior chamber; ACAID, anterior chamber–associated immune deviation; BMAPCs, bone marrow–derived APCs; DTH, delayed-type hypersensitivity; EGF, epidermal growth factor; EMR, EGF module–containing mucin-like hormone receptor; ES, embryonic stem; LAT, local adoptive transfer; PE, phycoerythrin; PEC, peritoneal exudate cells; TM7, seven-span transmembrane; T reg, regulatory T.

As professional phagocytes and APCs, macrophages are important in the innate as well as the adaptive immune system. In addition, macrophages also play a central role in tissue homeostasis, eliminating dying cells and nursing hemopoietic precursor cells (1). Many of their important functions are performed by cell-surface proteins, such as the complement receptors, the mannose receptor, and scavenger receptors (2, 3). The F4/80 glycoprotein, identified more than 20 years ago as the antigen for the F4/80 mAb, has been established as one of the most specific cell-surface markers for murine macrophages (4, 5). F4/80 is highly and constitutively expressed on most resident tissue macrophages, including the red pulp macrophages in the spleen, microglia in the brain, Kupffer's cells in the liver, and Langerhans'

cells in the skin (6). Furthermore, the expression of F4/80 is tightly regulated according to the physiological status of cells. Thus, the precursor of tissue macrophages, the blood monocyte, is known to express less F4/80 than its mature counterparts (7). F4/80 is expressed at lower levels on activated macrophages isolated from bacille Calmette-Guérin–infected animals than on unstimulated resting macrophages (8). Similarly, F4/80 expression is down-regulated on macrophages in response to IFN-γ (9). F4/80 expression on Langerhans' cells decreases after they take up antigens and become migrating DCs; in lymph nodes and spleens, F4/80 is detected only on macrophages in T cell–independent areas (7). These studies have pointed to a function for F4/80 in selected tissue macrophage populations.

Analysis of the F4/80 protein sequence revealed an intriguing hybrid structure consisting of motifs of two different protein superfamilies

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(10, 11). The epidermal growth factor (EGF)-like domains are located at the extracellular NH_2 terminus, whereas a seven-span transmembrane (TM7) domain similar to the class B G-protein–coupled receptors is located at the COOH terminus. Hence, F4/80 is a prototypic member of the EGF-TM7 receptor family that includes EGF module–containing mucin-like hormone receptor (EMR)1, 2, 3, 4, EGF-TM7-latrophilin–related protein (ETL) and CD97 (6, 12). The EGF-like module, which often occurs as multiple tandem repeats, has been identified in a wide variety of extracellular proteins involved in adhesion, receptor–ligand interactions, extracellular matrix structure, and the determination of cell fate (13). The TM7/G-protein–coupled receptor domain, on the other hand, has been shown to mediate the signal transduction of an extensive array of exogenous stimuli including hormones, cytokines, peptides, amino acid derivatives, ions, neurotransmitters, and sensory stimuli such as photons, taste, and odorants (14, 15). A dual adhesion and signaling function has thus been suggested for the EGF-TM7 molecules in which the extracellular region is involved in protein–protein interaction with other cell-surface proteins and/or extracellular matrix proteins, triggering intracellular signaling through the TM7 domain. Consistent with this hypothesis, specific cellular ligands for the EGF-TM7 receptors have been reported. CD55 (decay accelerating factor, DAF) was identified as the cognate cellular receptor for CD97 (16). More recently, we have identified cell-surface chondroitin sulfate as a specific ligand for the largest isoform of EMR2 and CD97 (17). Similarly, the presence of putative cell-surface ligands for EMR3 and EMR4 has also been demonstrated (18, 19). It is therefore reasonable to suggest that F4/80 may have a similar cell adhesion function, which in turn might influence the development, distribution and functions of tissue macrophages in vivo.

Peripheral tolerance in the adult serves to prevent immune recognition by self-reactive lymphocytes as well as to prevent inappropriate immune reactivity. F4/80+ APCs have previously been shown to carry a tolerance-inducing signal in a model of peripheral immune tolerance known as anterior chamber–associated immune deviation (ACAID)(20–22). ACAID-induced peripheral tolerance is both local and peripheral in its effects and helps protect the delicate structure of the visual axis from the devastating effects of immunogenic inflammation. Foreign antigens that enter the eye are captured by indigenous APCs of the iris and ciliary body that then travel via the blood to the spleen where they facilitate antigen-specific tolerance. Both CD4+ and CD8+ T regulatory (T reg) cells are generated to suppress the afferent and efferent arms of the immune response, respectively. During ACAID induction, F4/80+ APCs appear first in the bloodstream and then in the splenic marginal zones where the production of macrophage inflammatory protein 2 by these APCs is critical to the recruitment of CD1d–restricted NK T cells for the induction of tolerance (23, 24). It has been shown that ACAID can be induced in naive mice with the adoptive transfer of as few as 20 F4/80+ APCs generated in vitro by treatment with TGF-β2 and antigen (22). As in the ACAID model, a low-dose oral tolerance model in mice generates CD8+ T reg cells capable of suppressing Th1 effector functions (25). In addition, as in the eyes, some indigenous macrophages in the Peyer's patches also express the F4/80 antigen (26). Because of the similarities in these two tolerance models, we postulated a direct role for the F4/80 molecule in the induction of peripheral tolerance. F4/80-deficient mice were generated via gene targeting to delineate the role of F4/80 in the development and distribution of tissue macrophages and to investigate whether F4/80 itself is involved in the induction of peripheral tolerance.

RESULTS
The normal development of macrophages in *F4/80*−/− mice
An *F4/80*-targeting construct was prepared by replacing an ∼1.0-kb HindIII–XbaI fragment containing the coding sequence of the first exon and the 5′-end of the first intron with a promoterless β-galactosidase/pGK-Neo cassette. The homologous regions in both arms of the targeting construct are a 1.8-kb fragment 5′ to the first exon and a 2.8-kb fragment containing the exon 2 and the 3′-end of intron 1 (Fig. 1 A). F4/80+/− animals were bred to produce mutant mice, which were identified by genotyping of tail DNA using Southern blot hybridization (Fig. 1 B). The presence of a 14-kb HindIII fragment detected by probe A and the absence of probe B–hybridized DNA fragments in F4/80−/− DNA samples indicated that homologous recombination had indeed occurred at the *F4/80* locus. This result was further verified by PCR genotyping analysis in which only the 300-bp fragments specific to the *F4/80*-targeted alleles, but not the WT allele–specific 600-bp fragments, were amplified in F4/80−/− DNA samples (Fig. 1 B). Northern blot hybridization and RT-PCR analyses showed that no F4/80 transcript was identified in the mutant animals (Fig. 1, C and D). Likewise, no F4/80 protein expression was identified in the F4/80−/− mice using both polyclonal and monoclonal anti-F4/80 antibodies (Abs) in immunohistochemistry and flow cytometry (Fig. 1 E and Table I). The complete absence of the F4/80 transcript and protein in F4/80−/− mice indicates a F4/80 null mutation. The F4/80−/− mice appear healthy, have normal bodyweight, and are fertile. Histological examination of various organs and tissues including bone marrow, liver, lung, kidney, heart, spleen, thymus, and lymph nodes from F4/80−/− animals failed to show any gross abnormalities (unpublished data). Flow cytometric analysis also indicated that the development of B and T lymphoid cells in the mutant mice is not affected (Table I and Figs. S1–S3, available at http://www.jem.org/cgi/content/full/jem.20042307/DC1). To investigate whether the characteristics of tissue macrophage populations were affected by the inactivation of the *F4/80* gene, the expression profiles of various macrophage-restricted cell-surface proteins, including the complement receptor 3, class-A scavenger receptor, macrosialin,
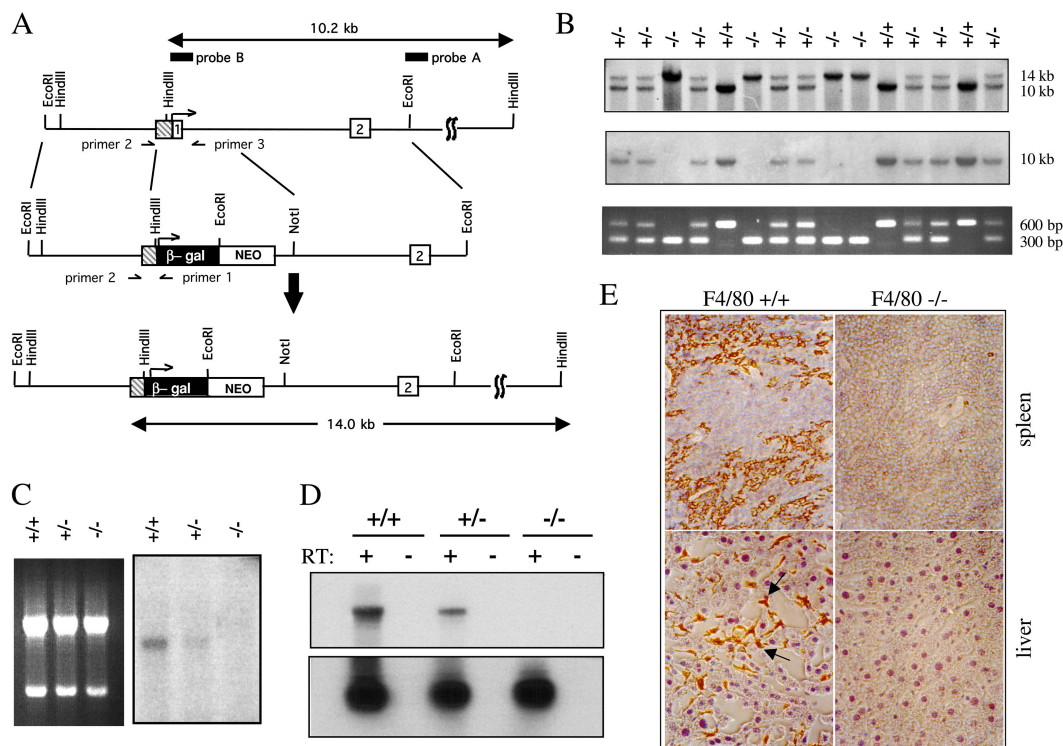
Figure 1. Gene targeting of the mouse *F4/80* gene. (A) Partial restriction maps of the mouse *F4/80* gene, the targeting construct, and the resulting targeted allele. The β-galactosidase and pGK-Neo gene cassettes are shown as black and white boxes, respectively. White boxes with numbers indicate exons. The 5′-UTR is represented by a stippled box. DNA probes A and B used for Southern blot analysis are shown. The expected HindIII fragments of the WT (10.2 kb) and the targeted alleles (14.0 kb) detected by probes A and B are also shown. Arrows with numbers indicate the positions and orientations of the primers used for the PCR genotyping reaction. (B) Southern blot and PCR genotyping. Tail DNA was digested with HindIII and hybridized with probe A (top) and probe B (middle). The same genomic DNA was analyzed using primers 1, 2, and 3. (C) Northern blot hybridization and (D) RT-PCR analysis of total RNA from thymus of F4/80$^{+/+}$, $+/-$, and $-/-$ animals using (C) the full-length F4/80 cDNA probe and (D) primers specific for the F4/80 (top) and Hprt (bottom) cDNAs. (E) mAb F4/80 staining in the spleens and livers (Kupffer's cells, arrow) of the F4/80$^{+/+}$ and $-/-$ animals. Magnification at 200 (spleen), 400 (liver).

and sialoadhesin, were examined. No difference in expression patterns of these molecules was observed between F4/80$^{-/-}$ mice and the WT controls (Fig. 2 and unpublished data). In addition, subpopulations of myeloid cells in blood and spleen, as detected by a panel of cell-surface markers, including CD11b, β-glucan receptor (Dectin-1), Gr-1, 7/4, and mannose receptor, were all comparable in WT and F4/80$^{-/-}$ mice (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20042307/DC1). Other phenotypic characterization between the WT and F4/80$^{-/-}$ mice was found to be similar and is summarized in Table I. For functional characterization, the binding and phagocytosis of zymosan and opsonized sheep red blood cells by thioglycollate-elicited macrophages were found to be similar in both WT and F4/80$^{-/-}$ cells (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20042307/DC1). The production of nitric oxide and inflammatory/antiinflammatory cytokines such as TNF-α, IL-10, and IL-12 in Bio-gel–elicited peritoneal macrophages treated with LPS and/or IFN-γ was measured. Again, no difference was observed in cells from the WT and F4/80$^{-/-}$ mice (Fig. S3, available at http://www.

jem.org/cgi/content/full/jem.20042307/DC1). Thus, we conclude that F4/80 is not necessary for the development and distribution of mouse tissue macrophage populations, and the inactivation of *F4/80* does not affect the general cellular functions of macrophages analyzed by conventional functional assays.

The role of F4/80 in the generation of CD8$^+$ T reg cells in an in vivo ACAID tolerance model

ACAID is a model for peripheral tolerance that is initiated by inoculation of antigen into the anterior chamber (a.c.) (for review see reference 27). Early reports showed that ACAID-inducing signals were carried from the eye to the spleen via APCs in the blood that expressed F4/80 protein (20, 21, 28). At that time, F4/80 Ab given in vivo was able to prevent the suppression of delayed-type hypersensitivity (DTH; ACAID) in experimental mice (20). However, the mechanism of the Ab treatment or the role of F4/80 protein in the model has not been studied further until now.

It is well established that a.c. inoculation of antigens leads to the development of splenic CD8$^+$ efferent T reg cells that
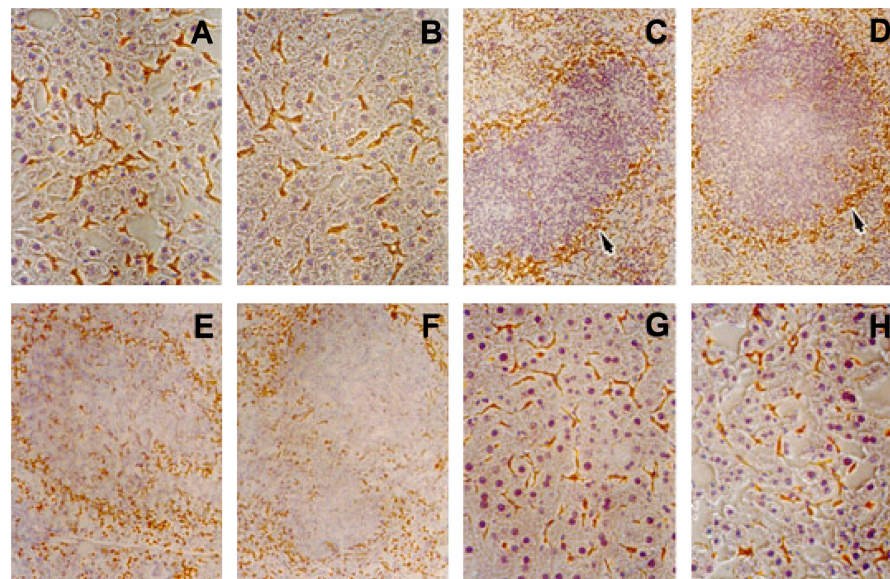
Figure 2.   Immunohistochemical analysis of the F4/80$^{-/-}$ macrophage. mAb 3D6 (anti-sialoadhesin) staining in the (A, B) livers and (C, D) and spleens of the (A, C) F4/80$^{+/+}$ and (B, D) $-/-$ animals. 3D6$^+$ Kupffer's cells and macrophages (brown) were found in the livers and the red pulp and marginal zone areas of spleens of both (A, C) F4/80$^{+/+}$ and (B, D) $-/-$ mice. The strongest 3D6 staining is on macrophages in the inner region of the marginal zone (marginal metallophils), forming a tight ring (arrow) around the white pulp (C, D). Magnification at 400 (A, B), 200 (C, D). (E, F) mAb 5C6 staining in the spleens and (G, H) mAb FA-11 staining in the livers of the (E, G) F4/80$^{+/+}$ and (F, H) $-/-$ animals. mAb FA-11 is specific for macrosialin (mouse CD68), whereas mAb 5C6 specifically recognizes type 3 complement receptor on myeloid cells. Magnification at 200 (E, F), 400 (G, H).

actively suppress an antigen-specific DTH response (29, 30). To test if the F4/80 protein had a functional role in the generation of ACAID-induced CD8$^+$ T reg cells, we inoculated OVA into the a.c. of WT and F4/80$^{-/-}$ mice, collected their splenic T cells, and examined their ability to suppress OVA-specific effector T cell function in a local adoptive transfer (LAT) assay. The LAT assay tests for efferent T reg cell function. It is established that the cells that affect this function are CD8$^+$ (30). In brief, enriched splenic T cells (regulatory cells) were collected from a.c.–inoculated mice and mixed equally with OVA–pulsed peritoneal exudate cells (PECs) (stimulators) and OVA–primed T cells (effectors). The cell mixture (1.5×10^5 cells total in 10 μl HBSS) was then injected intradermally to the ear pinnae of unmanipulated B6 mice. 24 h later, the ear swelling response was measured as an indicator of DTH. Splenic T cells from naive B6-WT mice did not suppress the adoptively transferred DTH response, whereas splenic T cells from WT mice inoculated with OVA (a.c.) did suppress the response (Fig. 3). In contrast, splenic T cells from OVA-inoculated (a.c.) F4/80$^{-/-}$

Table I. Summary of the phenotypic analysis of the F4/80 mutant mice

Genotype	+/+	+/−	−/−
Progeny no.	168/700 (24.0%)	358/700 (51.1%)	174/700 (24.9%)
RPC[a]	8.35 ± 0.54	8.07 ± 1.07	8.08 ± 1.48
TPC[b]	3.10 ± 0.26	ND	2.96 ± 0.15
Mac1$^+$ cells (RPC, TPC)[c]	37.5%, 78.4%	ND	37.7%, 76.5%
Mac1$^+$F4/80$^+$ cells (RPC, TPC)[c]	35.2%, 58.1%	ND	0.1%, 0.23%
CD4$^+$CD8$^+$ cells (spleen, thymus)[d]	0.81%, 73.1%	ND	1.20%, 74.2%
CD4$^+$CD8$^-$ cells (spleen, thymus)	16.3%, 17.6%	ND	15.9%, 16.8%
CD4$^-$CD8$^+$ cells (spleen, thymus)	4.50%, 4.10%	ND	4.90%, 3.80%
B220$^+$Thy1$^-$ cells[e]	67.9%	ND	68.3%
B220$^-$Thy1$^+$ cells	15.1%	ND	14.3%

[a]Total RPCs isolated by peritoneal lavage. Cells were counted by hemocytometer. Numbers given are cell number $\times$ 10^{-6}; $n = 8$.
[b]Total TPCs isolated by peritoneal lavage 4 days after thioglycollate injection. Numbers given are cell number $\times$ 10^{-7}; $n = 10$.
[c]Flow cytometric analysis of RPC and TPC using Mac1 and F4/80 mAbs. Numbers given are percentage of single- and double-positive cells from one representative experiment.
[d]Flow cytometric analysis of splenic and thymic cells using CD4 and CD8 mAbs.
[e]Flow cytometric analysis of spenic cells using B220 and Thy1 mAbs.
RPC, resident peritoneal cells; TPC, thioglycollate-elicited peritoneal cells.
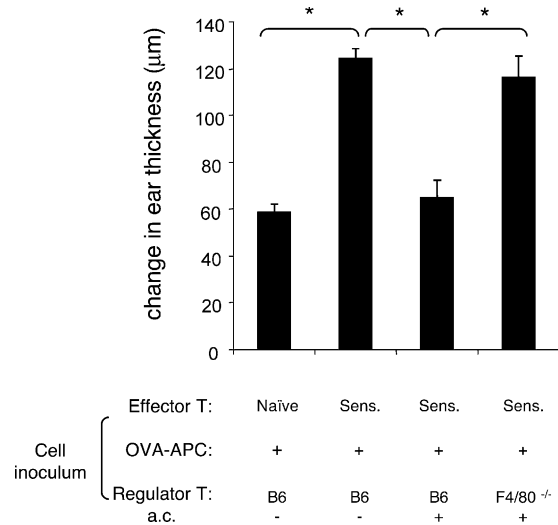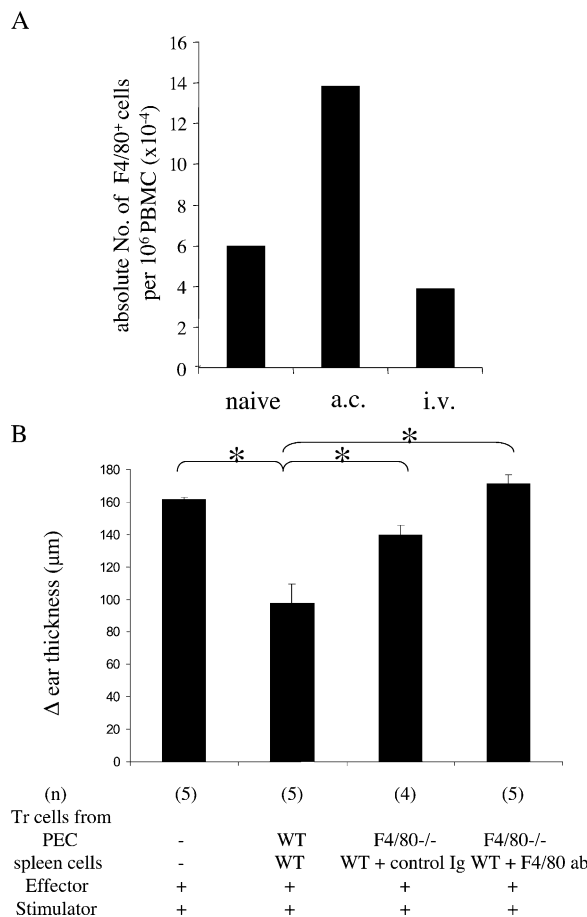
Figure 3. The role of F4/80 in the generation of regulatory T cells in vivo after a.c. inoculation of antigen. Enriched splenic T cells were collected from WT and F4/80$^{-/-}$ mice that had received a.c. OVA 7 d earlier and were mixed with WT, OVA-pulsed PECs, and OVA-primed T cells. The cell inoculum was given intradermally to the pinnae of naive B6 mice. Ear thickness was determined before and at 24 and 48 h after injection. Data are represented as mean change in ear thickness at 24 h after injection ± SEM. The composition of the cell inoculum is indicated under each bar in the graph. *, P < 0.01; n = 5/group.

mice failed to suppress the DTH response. Therefore, we concluded that peripheral tolerance that developed subsequent to a.c. inoculation of antigen failed in F4/80$^{-/-}$ mice because of a lack of efferent T reg cell development.

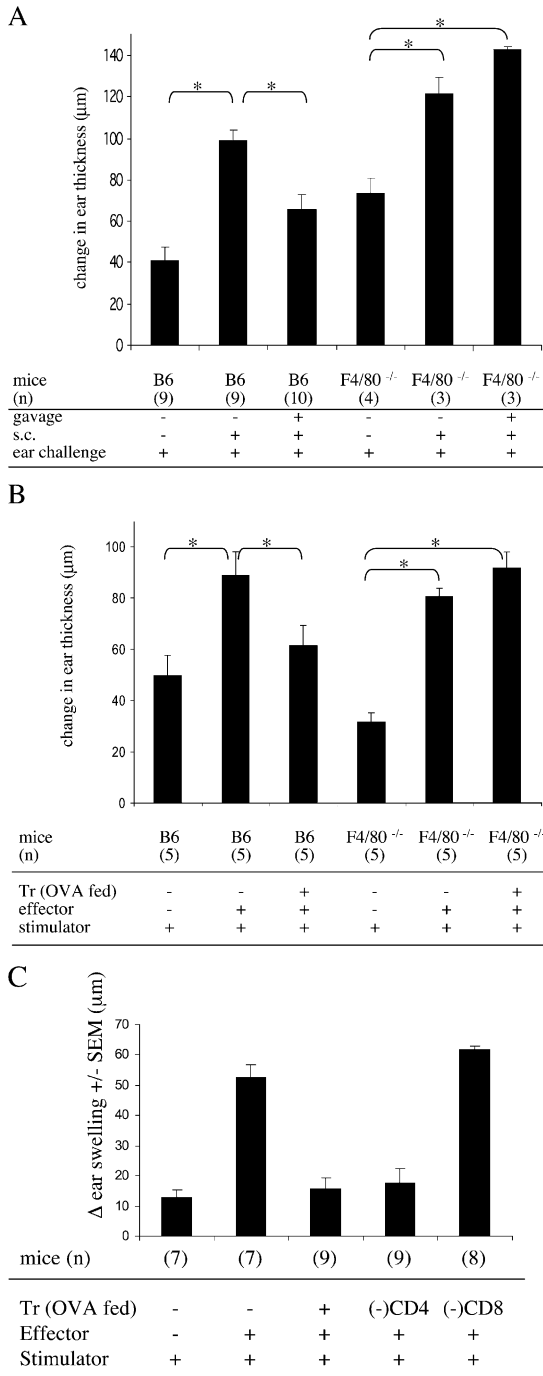## The role of F4/80 in the generation of T reg cells for peripheral tolerance in vitro

It is known that inoculation of antigen via the a.c. results in the appearance of tolerance-inducing F4/80$^+$ cells in the peripheral blood within 3 d (20, 31). To quantify the changes in the blood-borne F4/80$^+$ cell population, PBMCs from OVA-inoculated (either i.v. or a.c.) and naive mice were collected and stained with FITC-αF4/80 mAb. The F4/80$^+$ cell population constituted 6% of the total PBMC population (5.97 × 10^4 F4/80$^+$ cells/10^6 PBMCs) in naive mice but increased to nearly 14% of the total PBMC population (1.38 × 10^5 F4/80$^+$ cells/10^6 PBMCs) in a.c.-inoculated mice (Fig. 4 A). Unlike the a.c. route, i.v. inoculation of OVA was not associated with an increase in the number of circulating F4/80$^+$ cells (Fig. 4 A). Similarly, immunostaining of spleen sections from a.c.- but not i.v.- or s.c.-inoculated animals revealed a marked increase in the number of F4/80$^+$ cells forming cell clusters with NKT and conventional T cells in the splenic marginal zones (unpublished data).

We postulated that if APCs were to induce ACAID and generate efferent T reg cells, they must express F4/80 protein. To investigate whether the F4/80 molecule itself is involved in peripheral tolerance, a combination of PECs from

Figure 4. The role of F4/80 in the generation of regulatory T cells in vitro. (A) Assessment of F4/80$^+$ cells in the circulation after a.c. or i.v. inoculation of antigen. The bar graph represents the number of F4/80$^+$ cells per million blood mononuclear cells in naive mice and 3 d after a.c. or i.v. inoculation of OVA. The number of F4/80$^+$ cells was determined from four pooled, splenectomized mice for the a.c. and i.v. groups and five mice from a control group. The data shown are representative of two experiments in which similar results were obtained. (B) Generation of T reg cells with F4/80$^{-/-}$ APC in the presence or absence of anti F4/80 mAb. The bar graph shows the magnitude of ear swelling (ordinate) as a measure of DTH responses generated in naive B6 mice. Mice were given an intradermal ear injection of a cell mixture containing OVA-primed spleen cells and T reg cells. T reg cells were generated by coculturing naive spleen cells for 5 d with WT or F4/80$^{-/-}$ PECs that had been treated overnight with OVA with or without TGFβ2. Before the addition of the naive spleen cells, cells were cultured in presence or absence of anti-F4/80 mAb. Ear thickness was determined before ear pinnae injection and at 24 and 48 h after injection. *, P < 0.01; n = 5/group.

F4/80$^{-/-}$ and WT mice and anti-F4/80 mAb or a control rat IgG were used in an in vitro ACAID model of T reg cell generation (32). The in vitro ACAID model allowed us to manipulate the cells involved. Instead of collecting limited numbers of APCs from the peripheral blood of a.c.-inoculated animals, we generated an established surrogate ACAID APC to induce ACAID in vitro. In brief, thioglycollate-elicited PECs from either F4/80$^{-/-}$ or WT mice were cul-

A

mice (n)	B6 (9)	B6 (9)	B6 (10)	F4/80⁻/⁻ (4)	F4/80⁻/⁻ (3)	F4/80⁻/⁻ (3)
gavage	-	-	+	-	-	+
s.c.	-	+	+	-	+	+
ear challenge	+	+	+	+	+	+

B

mice (n)	B6 (5)	B6 (5)	B6 (5)	F4/80⁻/⁻ (5)	F4/80⁻/⁻ (5)	F4/80⁻/⁻ (5)
Tr (OVA fed)	-	-	+	-	-	+
effector	-	+	+	-	+	+
stimulator	+	+	+	+	+	+

C

mice (n)	(7)	(7)	(9)	(9)	(8)
Tr (OVA fed)	-	-	+	(-)CD4	(-)CD8
Effector	-	+	+	+	+
Stimulator	+	+	+	+	+

Figure 5. The role of F4/80 in the generation of regulatory T cells in vivo after low–dose oral gavage of antigen. (A) Low-dose oral toler-ance experiments in WT and F4/80⁻/⁻ mice: the ear swelling DTH response to OVA of F4/80⁻/⁻ mice immunized with OVA in CFA s.c. and ear challenged with OVA intradermally in the ear pinnae (positive DTH) versus those fed OVA (gavage). The treatment of the mice is indicated below the abscissa under each bar. Naive F4/80⁻/⁻ mice that received only OVA by ear challenge served as the negative DTH control. The number (n) and treatment of the mice are indicated along the abscissa under each bar graph. The change in ear thickness is listed on the ordinate. (B) Low-dose antigen gavage gener-ated an efferent T reg cell in WT but not in F4/80⁻/⁻mice. 7 d after OVA-gavage, enriched T cells from spleens were mixed with primed T cells

tured overnight in the presence or absence of OVA with or without TGF-β2. The cultured PECs were washed to re-move excess antigen and TGF-β. Because the PECs from the KO mice lack only the F4/80 protein, we expected them to respond as WT mice to the TGF-β2 treatment. In-deed TGF-β2–treated WT and F4/80⁻/⁻ PECs produce TGF-β (J. Stein-Streilein, unpublished data) and have the capacity to "educate" other APCs in the culture to acquire ACAID-inducing qualities. Therefore to control for the ability of TGF-β2–treated F4/80-deficient APCs influenc-ing F4/80⁺ splenic cells and to determine if there were a re-quirement for F4/80 expression on splenic APCs as well as the ACAID-inducing APCs, naive syngeneic spleen cells were added to the PECs and cultured for 5 d in the pres-ence or absence of either anti-F4/80 mAb or control rat IgG. At the end of the PEC–spleen cell coculture, nonad-herent cells were collected, enriched for T cells, and tested for efferent T reg cell function in the LAT DTH assay. T cells from cocultures with OVA-pulsed PECs from WT mice that had been pretreated with TGF-β2 (ACAID PECs) were found to suppress the DTH response signifi-cantly (Fig. 4 B). TGF-β2 is a major constituent of the aqueous humor (33, 34) and is one of the key molecules that endow resident eye-derived macrophages with tolero-genic potential (31). If the OVA-pulsed TGF-β2–treated PECs were harvested from F4/80⁻/⁻ mice and cultured with naive spleen cells in the presence of control rat IgG, there was partial suppression of DTH. However, when both treated F4/80⁻/⁻ PEC and naive spleen cells were cultured in the presence of anti-F4/80 mAb, there was no evidence of a functional efferent regulatory cell being generated (Fig. 4 B). Thus F4/80 protein must be expressed on the OVA-pulsed TGF-β–treated APCs and on APCs within the WT spleens to induce the T reg cell in vitro.

The role of F4/80 in the generation of CD8⁺ T reg cells in a low–dose oral tolerance model

To determine whether the role for the F4/80 molecule in the generation of CD8⁺ T reg cells is unique to ACAID, we tested the role of F4/80 in a low-dose oral tolerance model that is also known to generate CD8⁺ T reg cells (25). Al-

(effector) and OVA-pulsed APCs (stimulator) before being injected into the ear pinnae of naive mice. Phenotype of the cells in the mixture injected is indicated under the abscissa. The change in ear swelling is indicated on the ordinate. *, P ≤ 0.05. (C) LAT assay for efferent CD8⁺ T reg cells in WT mice that received antigen by gavage. 7 d after OVA-gavage, T cells were enriched from spleens and were either depleted of CD4⁺ T cells (GK1.5 and complement), depleted of CD8⁺ T cells (2.43 and complement), or kept un-manipulated. The enriched T cells were mixed with primed T cells (effector) and OVA-pulsed APCs (stimulator) before being injected into the ear pinnae of naive mice. The phenotype of the cells in the mixture injected is indi-cated under the abscissa. The change in ear swelling is indicated on the ordinate. *, P ≤ 0.05. Depletion of cells was confirmed using flow cytometry (not depicted).

though both high-dose and low-dose gavage of antigen can induce oral tolerance, only the low-dose procedure induces $CD8^+$ T reg cells. Briefly, WT and $F4/80^{-/-}$ mice were given 5 mg of OVA in saline by gavage. 1 wk later, experimental mice and a set of naive mice (positive control) were sensitized with OVA in CFA (s.c.). After another 7 d, the ear pinnae were inoculated intradermally with antigen-pulsed syngeneic PEC. The DTH response was suppressed in WT mice that received OVA by gavage but was apparent in both the positive control and the $F4/80^{-/-}$ mice that had received antigen by gavage (Fig. 5 A). To confirm that the tolerance response observed here is caused by the generation of efferent T reg cells, another low-dose oral tolerance experiment was performed. 7 d after mice received OVA by gavage, T cells were enriched from spleens and mixed with primed T cells (effectors) and OVA-pulsed APCs (stimulators) before being injected into the ear pinnae of naive mice. As expected, enriched T reg cells from OVA-gavaged WT

mice were able to suppress the DTH response, whereas the cells from OVA-gavaged $F4/80^{-/-}$ mice did not (Fig. 5 B). To demonstrate the phenotype of the T reg cell that was suppressing the DTH response, we repeated the LAT assays using enriched splenic T cells from OVA-gavaged mice that were unmanipulated or were depleted of either $CD4^+$ or $CD8^+$ T cells before mixing with the effector and stimulator cells. Cells were depleted of the $CD4^+$ or $CD8^+$ T cell subsets by incubation with GK1.5 or 2.43 mAb and complement, respectively. We observed that the enriched T cells were able to suppress the adoptively transferred response when the sample was unmanipulated or was depleted of $CD4^+$ T cells but not when the $CD8^+$ T cells were removed (Fig. 5 C). These observations confirm that, like ACAID, low-dose oral tolerance generates efferent $CD8^+$ T reg cells that efficiently suppress the effector phase of a DTH response. Thus the F4/80 protein is required for the process that leads to the generation of $CD8^+$ T reg cells in at least
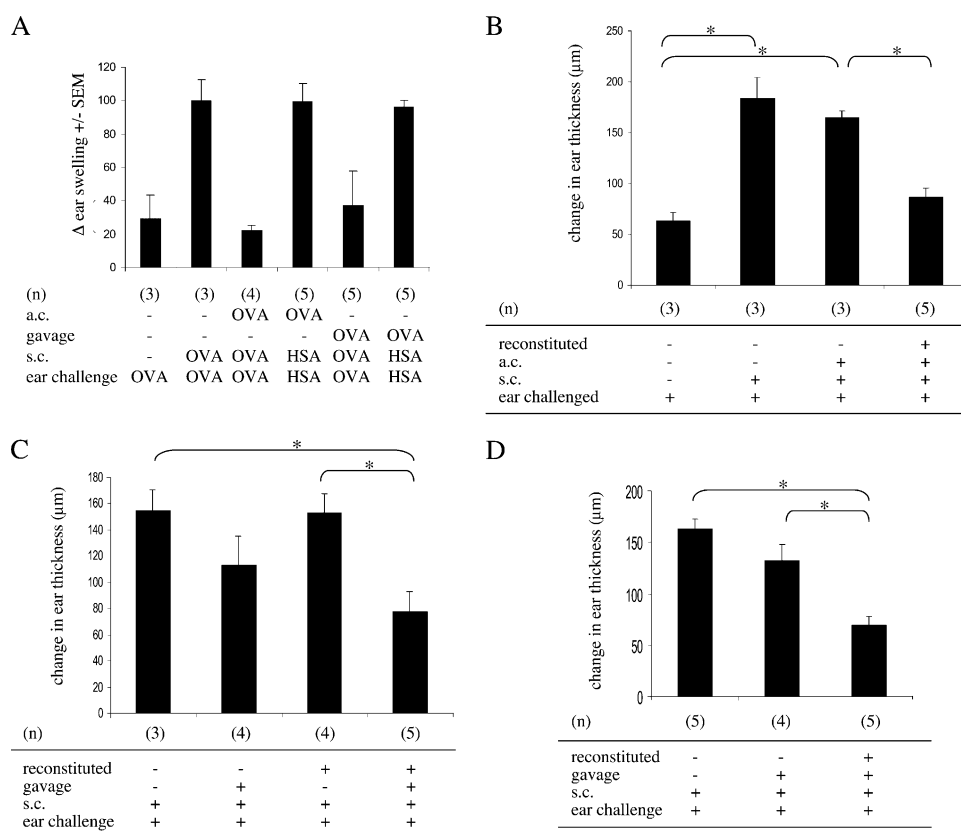
Figure 6.   Reconstitution of $F4/80^{-/-}$ mice with $F4/80^+$ APC restores peripheral tolerance. (A) To demonstrate antigen specificity of efferent suppression in two models of tolerance where animals received OVA either by intracameral inoculation or gavage before challenge in the ear with OVA or human serum albumin (HSA) (unrelated antigen). The change in ear swelling is on the ordinate, and the antigen regimen is displayed below bars under the abscissa. (B) In the ACAID tolerance model, $F4/80^{-/-}$ mice were reconstituted with 10^6 $F4/80^+$ bone marrow–derived APCs (BMAPCs) 1 d before inducing ACAID with OVA a.c. (C) In the low-dose oral tolerance model, $F4/80^{-/-}$ mice were reconstituted with 10^6 $F4/80^+$ BMAPCs. The experiment was repeated twice with similar results. (D) $F4/80^{-/-}$ mice were reconstituted with 10^6 $F4/80^+$ adherent spleen cells. The next day, mice were given OVA gavage, and 7 d later they were immunized with OVA s.c. After another week, they were challenged intradermally in the ear. DTH responses were measured as an increase in ear thickness. The treatment of the mice is indicated below the abscissa under each bar. The change in ear thickness is indicated on the ordinate. Significant ($P \leq 0.05$) differences between groups are indicated with an asterisk.

two models of peripheral tolerance: the ACAID and low-dose oral tolerance.

Reconstitution of the F4/80$^+$ cells in F4/80$^{-/-}$ mice and restoration of peripheral tolerance

Although it is well established that the efferent T reg cell is antigen specific (27), we confirmed here that in both ACAID and low-dose oral tolerance (Fig. 6 A) the suppression of DTH in the ear occurred only when the specific antigen inoculated into the eye or introduced into the gut was used for challenge (Fig. 6 A). To establish the role of F4/80 in both peripheral tolerance models unambiguously, we next restored the F4/80$^+$ cell populations in the F4/80$^{-/-}$ mice by transferring WT APCs into mutant mice before the beginning of either tolerance protocol. Separate groups of mice that were in the ACAID (Fig. 6 B) or low-dose oral tolerance (Fig. 6, C and D) protocol received 10^6 F4/80$^+$ bone marrow–derived APCs (BMAPCs) or F4/80$^+$ adherent splenic cells per mouse. All cells expressed F4/80 protein as determined by flow cytometry (unpublished data). F4/80$^{-/-}$ mice receiving no WT APCs were used as controls. We noticed that there was an insignificant suppression of DTH in the F4/80$^{-/-}$ mice that were gavaged but not reconstituted (Fig. 6, C and D). There may also be mechanisms that are F4/80-independent that contribute to suppression of DTH in oral tolerance. However, this possibility does not detract from our interpretation of Fig. 6, that both reconstitution protocols restore the ability of the previously deficient mice to respond to a.c. inoculation or gavage of OVA with the development of T reg cells.

DISCUSSION

To study the biological functions of the F4/80 protein, we have generated a mouse strain in which the *F4/80* gene is mutated and the synthesis of the F4/80 protein is disrupted (Fig. 1). No apparent abnormal phenotypes were identified in the F4/80$^{-/-}$ mice, indicating that F4/80 is dispensable for the development of mouse tissue macrophages. Schaller et al. recently also reported the generation of another strain of F4/80–deficient mice by knocking in Cre recombinase under the control of the F4/80 gene promoter (35). Consistent with our finding, the normal homeostatic development of macrophages was observed in F4/80$^{Cre/Cre}$ mice (35). Similarly, no obvious phenotypic changes were found in the F4/80$^{Cre/Cre}$ mice, even after infection with live *Listeria monocytogenes*.

Nevertheless, other reports have implicated F4/80 in macrophage functions in pathological and inflammatory situations. A functional requirement for F4/80 in the production of TNFα, IL-12, and IFN-γ after exposure of the mouse spleen cells to either heat-killed or viable *Listeria monocytogenes* has been demonstrated (36). In this model, F4/80 has been shown to be involved in direct cell–cell contact between macrophages and NK cells that results in cell activation and optimal cytokine production. Also noteworthy is the identification of an eye-derived F4/80$^+$ cell as the cell that carries the ACAID-inducing signal to the spleen (20,

21, 28, 31). Wilbanks et al. went on to show that in vivo treatment of a.c.-inoculated mice with F4/80 Ab prevented the suppression of DTH (ACAID) in the mice (20). As a result, F4/80 became a marker of the "eye-derived" APCs that were central to the induction of ACAID. Here, and in previous reports from our laboratory, we show that, after a.c. inoculation of the antigen, F4/80$^+$ cells accumulate in the marginal zone of the spleen, forming clusters with NK T cells and conventional T cells (24). This observation is in keeping with other observations that F4/80$^+$ cells, although present abundantly in the red pulp (37), are normally absent in T cell areas and in the marginal zones (7). If the F4/80 protein becomes a marker for tolerogenic APCs, its function may be relevant to our hypothesis that tolerance induction occurs in non–T cell areas of the secondary lymphoid organs. Future studies regarding the function of F4/80 protein during tolerance induction will help us to explore this idea.

The interaction between F4/80$^+$CD1d$^+$ tolerogenic APCs and NK T cells was essential for the development of CD8$^+$ T reg cells capable of suppressing DTH toward eye-derived antigens (23). These results prompted us to ask whether the F4/80 molecule itself plays a direct role in the induction of peripheral tolerance. We showed here that not only do F4/80$^+$ APCs increase in the blood and then in the spleen after an inoculation of antigen into the eye, but that the F4/80 molecule is important for the generation of efferent T reg cells (Figs. 3 and 4). It is well established in the ACAID literature that the efferent T reg cell is CD8$^+$ and antigen specific (30, 38). This is further supported by our report that the ACAID efferent regulatory T cell is easily generated in class II–deficient mice, a strain that has no conventional CD4$^+$ T cells (39).

Additionally, we showed that the requirement of F4/80 is not unique to CD8$^+$ T reg cell generation in the ACAID model; F4/80 is also needed for CD8$^+$ T reg cell induction in low-dose oral tolerance (Fig. 5). This is the first report of a role for the F4/80 molecule in the induction of CD8$^+$ T reg cells and in peripheral tolerance. Although oral tolerance shares some properties with ACAID, there are also differences between the two models. First, the environment of the gut is not generally considered to be immune privileged, because it is not a site that normally accepts foreign grafts (27). However, the gut does have a complicated cascade of events that prevents certain types of immune responses to ingested protein antigens. One of the similarities is that both the eye and the gut contain large amounts of TGF-β (34, 40–42). Moreover, as in ACAID we observed that low-dose oral tolerance is dependent on the presence of the invariant NK T cell because Jα18-deficient mice that lack the invariant NKT cell are unable to acquire tolerance after low-dose antigen gavage (unpublished data) (43). Class II–deficient mice are still able to generate T cells capable of suppressing the DTH effector function of T cells in both models. Also, we show here that the efferent regulatory function is antigen specific in both models of tolerance.

Although the gut/intestine is not considered to be an immune privileged site (27), an essential feature of the intestinal immune system is its ability to discriminate between harmful and harmless foreign proteins (44). Under normal conditions, the individual is tolerant to soluble food antigens but is capable of mounting effective immune responses when confronted with invasive organisms. The mechanism of oral tolerance is unclear and may result from deletion, anergy, or active regulation of immune effector cells (45, 46). It is thought that the diverse local APCs present in the intestine play a major role in determining the T cell response to antigen (47). It is believed that mucosal DCs may in their native state preferentially behave as tolerogenic APCs. Many of the APCs in Peyer's patches constitutively secrete immunosuppressive cytokines such as IL-10 (48, 49). It is interesting that the APCs in the Peyer's patches also express F4/80 protein (26). In keeping with the idea that the F4/80 molecule may identify tolerogenic APCs, Suter et al. recently reported that the majority of DCs in the brain/central nervous system, another immune privileged site where tolerance is promoted, also expressed F4/80 (50). Our studies here show that the F4/80 protein on APCs is needed for the generation of the CD8+ T reg cells in oral tolerance and provide evidence that suppression of DTH responses may be transferred with CD8+ T cells after antigen gavage (Fig. 5 C). Currently, we are characterizing the surface phenotype of the F4/80+ tolerogenic APCs in the ACAID model. We find that the F4/80+ APCs lack coreceptors for T cell activation and do not up-regulate CCR-7, the chemokine receptor that guides APCs to the T cell areas of the secondary lymphoid organs (J. Zhang-Hoover, J. Stein-Streilein, unpublished data).

Given the lack of a known ligand for F4/80, it remains difficult to define a molecular mechanism mediating the differentiation of CD8+ T reg cells. It is plausible that F4/80 serves to stabilize adhesive interactions between APCs and other cell types including NK cells or NK T cells. This idea is consistent with the observation that Langerhans' cells down-regulate F4/80 expression after taking up antigen to become veiled cells that migrate via the lymph to regional nodes. Also supporting an adhesive function for the F4/80 molecule is the observation that circulating blood monocytes express lower cell surface levels of F4/80 than do tissue macrophages (7). Alternatively, it is possible that the F4/80 molecule, once engaged by its ligand, participates in intracellular signaling events that lead to APC cytokine production that facilitates T cell differentiation. The development of a functional soluble form of the F4/80 ligand may allow us to determine whether signaling through the F4/80 molecule triggers activation, differentiation, or cytokine production by APCs.

In this report we show for the first time that the F4/80 molecule is required for the differentiation of antigen–specific CD8+ T reg cells, a process that requires direct cellular interaction of CD1d+ APCs with NK T cells. There are many correlations with NK T cell activation in self-tolerance and their deficiencies in autoimmune diseases such as diabe-tes, multiple sclerosis, and lupus (51, 52). Identifying the cellular and molecular mechanisms used by the F4/80 molecule to facilitate the differentiation of T reg cells will contribute to our understanding of the processes involved in immunological tolerance and/or its breakdown.

MATERIALS AND METHODS

Antibodies. For the in vitro assays, anti-F4/80 mAb was purified from F4/80 hybridoma supernatants and used at a concentration of 10 µg/ml. Ab 3D6, 2F8, 5C6, and FA-11 were specific to sialoadhesin, macrophage class-A scavenger receptor, the type 3 complement receptor, and macrosialin, respectively. mAbs used for flow cytometric and tissue staining analysis were phycoerythrin (PE)-conjugated anti–mouse CD4 Ab (0.5 mg/ml; Caltag Laboratories), FITC-conjugated anti–mouse CD8 Ab (0.5 mg/ml; Caltag Laboratories), FITC-conjugated anti–mouse Thy-1.2 Ab (1 mg/ml; Caltag Laboratories), PE- and Rhodamine-conjugated anti–mouse B220 Ab, Rhodamine-conjugated anti–mouse CD3 Ab (BD Biosciences), FITC-conjugated anti–mouse Mac-1 Ab (BD Biosciences), PE-conjugated anti–mouse F4/80 Ab (Serotec), and PE- or FITC-conjugated rat IgG$_{2b}$(K) Ab (BD Biosciences) as isotype control. Anti–mouse FcγII/IIIR Ab was used to block nonspecific binding of Abs through Fc.

Construction of the F4/80 targeting vector. To make the *F4/80* gene-targeting construct, a 1.8-kb EcoRI-HindIII fragment containing the 5′-UTR of the *F4/80* gene was subcloned into the NotI and HindIII sites of the pSA β-galactosidase–Neo vector (53). A 2.8-kb XbaI–EcoRI fragment containing exon 2 of the *F4/80* gene was then inserted into the previous construct through the XbaI and KpnI sites. The completed construct, pF4/80 β-galactosidase, resulting in the deletion of the exon 1 coding sequence of the *F4/80* gene, is shown in Fig. 1 A.

Mice. The targeting construct, pF4/80–β-galactosidase, was linearized with KpnI and electroporated into CJ7 mouse embryonic stem (ES) cells as described previously (54). Genomic DNA isolated from the resulting ES cell clones was analyzed by Southern blot hybridization using HindIII digestion and a ^{32}P-labeled 0.2 kb EcoRI–BamHI fragment (probe A in Fig. 1 A). Microinjection of targeted ES cells to C57BL/6J blastocysts and the generation of heterozygous and homozygous mice were performed as described previously (54). Gene targeting was confirmed by Southern blot analysis of tail DNA and the PCR genotyping reaction using a primer specific for the β-galactosidase gene (primer 1: 5′-TCCGCCATGTCAC-AGATCAT-3′) and two *F4/80* gene-specific primers (primer 2: 5′-GGAAGGAAATGGAGAGAAAG-3′, and primer 3: 5′-GAAGATCT-ACCCTGGTGAAT-3′) (Fig. 1). The F4/80$^{-/-}$ mice were backcrossed to C57BL/6 (B6-WT) background for more than 10 generations (B6-F4/80$^{-/-}$). All animals were treated humanely and in accordance with the guidelines set forth by the Animal Care and Use Committee of Sir William Dunn School of Pathology, Schepens Eye Research Institute, and the National Institute of Health.

Northern blot hybridization and RT-PCR analysis. Total RNA isolated from thymus, spleen, and liver of 5-wk-old F4/80 +/+, +/−, and −/− mice was analyzed by Northern blot hybridization using a full-length F4/80 cDNA probe and by RT-PCR using gene-specific primers (5′-AGGACTG-GAAGCCCATAGCCAA-3′, and 5′-GCATCTAGCAATGGACAGCTG-3′) as described previously (11). Primers for the hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) gene were included as an internal control.

Immunohistochemical and flow cytometric analysis. Immunohistochemical staining of tissue sections of F4/80 +/+, +/−, and −/− mice was performed as described (55). Flow cytometric analyses were done on an EPICS XL flow cytometer (Beckman Coulter). The percent of F4/80+ cells was determined after gating on the entire viable PBMC population. The

absolute number of F4/80$^+$ cells per 10^6 PBMCs was calculated according to the percentage of F4/80$^+$ cells found in the total number of cells obtained per milliliter of blood.

Cell enrichment. PBMCs used in the flow cytometric analyses were isolated from heparinized venous blood obtained at 3 and 7 d after a.c. inoculation of antigen. PBMCs were enriched by density-gradient centrifugation using Lympholyte-M (Cedarlane Laboratories, Ltd.). Splenic T cells used for the LAT assay (described below) were enriched using IMMULAN T cell enrichment columns (Biotecx Laboratories, Inc.). Resident peritoneal macrophages (RPMϕ) and thioglycollate-elicited peritoneal macrophages (TPMϕ) were obtained from 8–10-wk-old mice. TPMϕ were harvested from animals injected i.p. with 1.0 ml of 3.0% (wt/vol) sterile Brewer's thioglycollate medium (Difco) 4 d earlier.

Antigen inoculations. For i.v. and s.c. administration of antigen, each mouse received 50 μg OVA in 100 μl HBSS via the tail vein or subcutaneously, respectively. For a.c. inoculation of antigens, mice were anesthetized with ketamine/xylazine as described previously (23, 24). All OVA solutions were passed through DetoxiGelAffinityPakpolymyxin B columns (Pierce Chemical Co.) to remove contaminating endotoxin/LPS.

LAT assay. LAT was used to test for the presence or absence of regulatory CD8$^+$ T cells as described previously (23, 24). In brief, OVA-primed effector T cells were generated by immunizing C57BL/6 mice with OVA in CFA (Sigma-Aldrich). 7 d later the spleens were collected and enriched for T cells. T reg cells were enriched from the spleens of either B6-WT or B6-F4/80$^{-/-}$ mice that had received OVA or HBSS (a.c.) 7 d earlier. Stimulator cells were obtained by culturing thioglycollate-elicited PECs with 5 ng/ml OVA overnight. Stimulator, effector, and regulatory cells (5 × 10^5 each) were resuspended in 10 μl HBSS and injected intradermally into the pinnae of naive mice. The change in ear thickness was measured at 24 and 48 h after challenge using an engineer's micrometer (Mitutoyo/MTI Corporation).

Induction of oral tolerance and DTH assay. Tolerance induction, sensitization, and challenge were performed using OVA that had been depleted of contaminating endotoxin by passage over a Detoxi Gel column (Pierce Chemical Co.). Oral tolerance was induced by feeding mice OVA (5 mg in 200 μl PBS) by gavage after mice had been deprived of food for 4–6 h. 7 d after induction of oral tolerance, mice were sensitized s.c. with 150 μl of an emulsion of endotoxin-free OVA (2.0 mg/ml in PBS) and CFA. 1 wk after sensitization, mice were challenged intradermally with OVA-pulsed PECs (2.0 × 10^5 cells in 10 μl PBS) into the pinnae. The change in the ear thickness was measured 24 h later as an indication of the DTH response.

Preparation of BMAPCs and adherent spleen cells for reconstitution experiments. BMAPCs were generated by culturing bone marrow cells in L929-conditioned growth medium (10% L929-conditioned medium, 10% horse serum, 10% controlled-process serum replacement type 3) for 6 d (56). The cells were collected; cell viability was >90%. Collected APCs were washed and resuspended in HBSS before i.v. inoculation of 10^6 cells per mouse. Spleens were removed from killed mice, dissociated into single cell suspensions, and layered on Petri dishes at a concentration of 2 × 10^6 cells/ml in HBSS in 10% FBS. After a 90-min incubation at 37°C, the nonadherent cells were washed away, and the adherent cells were harvested. The expression of F4/80 on BMAPCs and adherent spleen cells was confirmed by immunostaining and flow cytometry (unpublished data).

Statistical analyses. All statistical analyses were performed by analysis of variance with Neuman-Keuls post hoc analyses. Significance was determined at $P < 0.05$.

Online supplemental material. Fig. S1 depicts flow cytometric characterization of the myeloid cell populations in peripheral blood (A) and spleen (B) of WT and F4/80 KO mice. Fig. S2 shows a quantitative phagocytic assay of the WT and F4/80 KO thioglycollate-elicited microphage. Fig. S3 depicts NO and cytokine production of Bio-gel–elicited macrophages from the WT and F4/80 KO mice (Fig. 3). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20042307/DC1.

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