Foxp3-positive Macrophages Display Immunosuppressive Properties and Promote Tumor Growth

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Foxp3-positive macrophages display immunosuppressive properties and promote tumor growth

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Regulatory T cells (T reg cells) are characterized by the expression of the forkhead lineage-specific transcription factor Foxp3, and their main function is to suppress T cells. While evaluating T reg cells, we identified a population of Foxp3-positive cells that were CD11b+F4/80+CD68+, indicating macrophage origin. These cells were observed in spleen, lymph nodes, bone marrow, thymus, liver, and other tissues of naïve animals. To characterize this subpopulation of macrophages, we devised a strategy to purify CD11b+F4/80+Foxp3− macrophages using Foxp3-GFP mice. Analysis of CD11b+F4/80+Foxp3+ macrophage function indicated that these cells inhibited the proliferation of T cells, whereas Foxp3− macrophages did not. Suppression of T cell proliferation was mediated through soluble factors. Foxp3− macrophages acquired Foxp3 expression after activation, which conferred inhibitory properties that were indistinguishable from natural Foxp3+ macrophages. The cytokine and transcriptional profiles of Foxp3+ macrophages were distinct from those of Foxp3− macrophages, indicating that these cells have different biological functions. Functional in vivo analyses indicated that CD11b+F4/80+Foxp3+ macrophages are important in tumor promotion and the induction of T reg cell conversion. For the first time, these studies demonstrate the existence of a distinct subpopulation of naturally occurring macrophage regulatory cells in which expression of Foxp3 correlates with suppressive function.

Abbreviations used: CTLA-4, cytotoxic T cell–associated antigen 4; DT, diphtheria toxin; GITR, glucocorticoid-induced TNF receptor family-related gene/protein; iNOS, inducible NO synthase; MDSC, myeloid-derived suppressor cell; PDGF, platelet-derived growth factor; PGE2, prostaglandin E2; T reg cell, regulatory T cell; VEGF, vascular endothelial growth factor.
Currently, there are no defined cellular markers that can distinguish activator versus suppressor subpopulations of MO. Because MOs have regulatory properties that are found only under inflammatory conditions, it is unclear whether MO could display regulatory function constitutively in the absence of inflammatory or polarizing stimuli. In the present studies, we have identified a previously uncharacterized naturally occurring subpopulation of MO (CD11b⁺F4/80⁺) that expresses Foxp3. Overall, our results indicate that CD11b⁺F4/80⁺Foxp3⁺ cells have immunoregulatory properties that inhibit T cells and contribute toward tumor progression. For the first time, these studies describe a previously unidentified population of naturally occurring CD11b⁺F4/80⁺Foxp3⁺ macrophage regulatory cells that may contribute to the regulation and maintenance of homeostatic balance of the immune system.

**RESULTS**

Identification of CD11b⁺Foxp3⁺ cells

During the course of our studies evaluating CD4⁺ T reg cells, we observed a population of CD11b⁺ cells that expressed Foxp3 in spleen from C57BL/6 mice (Fig. 1 A). Further evaluations revealed that CD11b⁺Foxp3⁺ cells were also present in bone marrow, thymus, lymph node, and liver (Fig. 1 A) and other organs such as lung and peripheral blood (not depicted). The CD11b⁺Foxp3⁺ cells represent 0.2–1% of the total number of cells in thymus, spleen and lymph nodes, whereas higher percentages were observed in bone marrow (∼5–6%) and liver (∼2–2.5%). To further define this CD11b⁺Foxp3⁺ population, bone marrow, thymus, spleen, lymph node, and liver tissues were stained for CD4/Foxp3, CD8/Foxp3, and CD11c/Foxp3 from spleen cells of C57BL/6 mice. (B) Percentages of double-positive CD11b/Foxp3 cells were determined from bone marrow and spleen cells of RAG-1 KO mice. Positive CD11b/Foxp3⁺ cells were gated and expression of F4/80 was determined. All data represent one of at least three separate experiments.
Isolation of CD11b^+ F4/80^+ Foxp3^+ cells from Foxp3-GFP mice

Foxp3-GFP transgenic mice were used to isolate and characterize CD11b^+ F4/80^+ Foxp3^+ cells. Staining of bone marrow (Fig. 2 A) from Foxp3-GFP mice indicates that CD11b^+ F4/80^+ CD68^+ GFP(Foxp3)^+ cells are present in similar proportions as in C57BL/6 mice. For functional in vitro analyses of CD11b^+ F4/80^+ GFP(Foxp3)^+ cells, we devised a strategy where bone marrow (or spleen) cells from Foxp3-GFP mice were depleted of T and B cells and stained for CD11b and F4/80. CD11b^+ F4/80^+ cells were gated (Fig. 2 B, fuchsia) and sorted based on the GFP-Foxp3 expression into Foxp3^+ and Foxp3^- cells. CD4^+ cells (sorted MO will be called F4/80^+ Foxp3^- or F4/80^+ Foxp3^+). To further enrich and eliminate traces of any contaminating cells in the F4/80^+ Foxp3^- and F4/80^+ Foxp3^+ populations, cells were sorted twice. The high purity of the two sorted populations was confirmed by flow cytometry (Fig. 2 B). Foxp3 levels were examined by PCR (Fig. 2 C), indicating that sorted F4/80^+ Foxp3^- cells, but not sorted F4/80^+ Foxp3^- cells, express Foxp3 to the same degree as sorted CD4^+ Foxp3^+ cells. To further confirm that F4/80^+ Foxp3^+ cells were of MO origin and have MO function, morphological and phagocytic analyses were performed. As shown in Fig. 3 A, single-sorted F4/80^+ Foxp3^+ and F4/80^+ Foxp3^- cells have morphological characteristics similar to those of MO but clearly distinct from those of CD4^+ T cells. To evaluate the phagocytic capacity of F4/80^+ Foxp3^+ and F4/80^+ Foxp3^- cells, they were incubated with Alexa Fluor 700-labeled beads at different cell/bead ratios for 4 or 24 h. The data indicate that both F4/80^+ Foxp3^+ and F4/80^+ Foxp3^- cells have a similar capacity to phagocytose the beads, confirming that they are both of MO origin (Fig. 3 B).

Phenotypic characterization of CD11b^+ F4/80^+ Foxp3^+ cells from Foxp3-GFP mice

After sorting the Foxp3^+ and Foxp3^- MO from bone marrow, we observed that the CD11b^+ Foxp3^- cells were CD11b^hi F4/80^low, whereas CD11b^+ Foxp3^+ cells were CD11b^low F4/80^hi (Fig. 4 A). T reg cells are characterized by the expression of cytotoxic T cell–associated antigen 4 (CTLA-4; Takahashi et al., 1998) and glucocorticoid-induced TNF receptor family-related gene/protein (GITR; Shimizu et al., 2002), whereas MDSCs are defined by the expression of CD11b and GR–1 cellular markers. Recently, Gallina et al. (2006) showed that the presence of IL-4Rα on a subset of CD11b^+ cells appeared to be critical for their suppressive activity. Therefore, we stained for GR–1, CTLA-4, GITR, and IL-4R. Our results show that the percentage of double-sorted F4/80^+ Foxp3^- cells expressing GITR, IL-4R, and CTLA-4 is threefold higher compared with
lack suppressive activity, whereas freshly isolated Foxp3⁺ T reg cells are inhibitory. We investigated whether freshly isolated single- or double-sorted F4/80⁺ Foxp3⁺ and F4/80⁺Foxp3⁻ MO were naturally inhibitory. As shown in Fig. 5 A, single- or double-sorted F4/80⁺Foxp3⁻ cells (Fig. 4 B). Similar results were also observed in F4/80⁺Foxp3⁻ (Fig. S1 A) or F4/80⁺ Foxp3⁺ (Fig. S1 B) cells from spleen. In contrast, both populations of F4/80⁺Foxp3⁻ and F4/80⁺Foxp3⁺ cells expressed similar levels of GR-1. These results indicate that F4/80⁺Foxp3⁺ and F4/80⁺Foxp3⁻ cells differentially express cellular markers that might contribute to their function.

Suppressive characterization of F4/80⁺Foxp3⁺ cells

Previous studies have shown that freshly isolated CD11b⁺ Gr-1⁺ or CD11b⁺F4/80⁺ cells from naive animals typically
cells, but not F4/80-Foxp3- cells, inhibited the proliferation of CD4+Foxp3- T cells (Fig. 5 C). These results confirm that Foxp3+ MO are able to inhibit T cells in the absence of T reg cells. We also confirmed that F4/80+Foxp3+ cells, but not F4/80-Foxp3- cells, inhibited the proliferation of antigen-activated CD4+ (OTII) and CD8+ (OTI) cells (Fig. S2A and B).

To validate that Foxp3 is critical in imparting immune-suppressive capabilities to CD11b+F4/80+ cells, the expression of Foxp3 was depleted in single-sorted F4/80+Foxp3+ cells by small interfering (si) RNA (Fig. 5 D). The results show that after depletion of Foxp3 expression in F4/80+Foxp3+ cells, the cells lost their capacity to inhibit proliferation of T cells, confirming that Foxp3 is directly responsible for conferring suppressive capabilities to this subpopulation of MO (Fig. 5 E).

To evaluate whether factors secreted by F4/80+Foxp3+ cells could also have suppressive capacity, supernatants from single-sorted Foxp3+ and Foxp3- MO were evaluated for their ability to inhibit the proliferation of CD4+ T cells. Our results indicate that only supernatants from Foxp3+ MO inhibited the proliferation of CD4+ T cells (Fig. 6 A). To define which factor produced by F4/80+Foxp3+ cells inhibited the proliferation of T cells, supernatants from these cells were incubated with neutralizing antibodies against IL-10, TGF-β1, TGF-β2, and prostaglandin E2 (PGE2). Our results show that only anti-PGE2 reverses the suppressive effect of the supernatant (Fig. 6 B), indicating that PGE2 is the inhibitory factor produced by F4/80+Foxp3+ cells. The levels of PGE2 secretion from F4/80+Foxp3+ and F4/80+Foxp3- cells were evaluated. Our data indicate that F4/80+Foxp3+ cells secrete large
amounts of PGE₂, whereas the secretion of PGE₂ by F4/80⁺Foxp3⁻ cells is minimal or nonexistent (Fig. 6 C). We wanted to assess whether F4/80⁺Foxp3⁺ cells induce suppression by cell–cell contact mechanisms. We used a transwell system where single-sorted F4/80⁺Foxp3⁺ cells were added at the indicated ratios to the upper chamber, and purified CD4⁺ T cells were placed in the bottom chamber and stimulated with anti-CD3/anti-CD28 plus irradiated APCs. As shown in Fig. 6 D, T cell proliferation was substantially inhibited at the highest ratio (1:1) between Foxp3⁺ MØ/CD4⁺ T cells, and less inhibition was observed at lower ratios. These results indicate that suppression was mediated by the secretion of soluble factors and is independent of cell-to-cell contact.

**Induction of CD11b⁺F4/80⁺Foxp3⁺ from CD11b⁺F4/80⁻Foxp3⁻ cells**

It is well established that CD4⁺ effector T cells that do not express Foxp3 can be induced to express Foxp3 by the addition of TGF-β (Chen et al., 2003). Such induced T reg cells have the same capacity as natural T reg cells to inhibit other cells (Chen et al., 2003). We evaluated whether F4/80⁺Foxp3⁻ cells could be converted to express Foxp3. Double-sorted F4/80⁻Foxp3⁻ cells from bone marrow were incubated in the presence of LPS, CpG, TGF-β, or vascular endothelial growth factor (VEGF) for 3 d. As shown in Fig. 7 A, the addition of LPS (42%), CpG (39%), TGF-β (50%), and VEGF (43%) to the cultures induces the expression of Foxp3 in these cells. Similar results were observed in F4/80⁻Foxp3⁻ cells from spleen samples (Fig. 53). To make sure that the de novo expression of Foxp3 is not the result of an expansion of a contaminating population of MØ-expressing Foxp3, we evaluated the proliferative capacity of double-sorted F4/80⁺Foxp3⁻ and F4/80⁺Foxp3⁺ cells after stimulation with LPS, F4/80⁺ Foxp3⁻ and F4/80⁺Foxp3⁺ cells do not proliferate after culturing them in complete medium alone or in the presence of LPS (Fig. 7 B). These cells are only able to proliferate if they are in the presence of macrophage (M) CSF (Fig. 7 B). We also characterized the phenotype of the double-sorted induced F4/80⁺Foxp3⁺ cells (Fig. 7 C). As observed with the natural F4/80⁺Foxp3⁺ cells, induced F4/80⁺Foxp3⁺ cells expressed higher levels of GITR, IL-4R, and CTLA-4 compared with LPS-treated F4/80⁺Foxp3⁻ cells (Fig. 7 C), and no difference was observed in GR-1 expression. Furthermore, induced F4/80⁺Foxp3⁺ cells were able to inhibit the proliferation of CD4⁺ T cells, whereas no significant inhibition was observed with LPS-treated F4/80⁺Foxp3⁻ cells (Fig. 7 D). Collectively, these results demonstrate that induced F4/80⁺Foxp3⁺ cells behave similarly to naturally occurring F4/80⁺Foxp3⁺ cells, paralleling the manner in which induced T reg cells behave like natural T reg cells.

**Global transcriptional analysis of CD11b⁺F4/80⁺Foxp3⁻ and CD11b⁺F4/80⁺Foxp3⁺ cells**

To further characterize and evaluate whether other biological differences exist between F4/80⁺Foxp3⁻ and F4/80⁺Foxp3⁺ cells, gene expression profiles from these populations were studied. For these experiments, we used double-sorted F4/80⁺ Foxp3⁻ and F4/80⁺Foxp3⁺ cells. We observed that 3,963 genes were differentially expressed (P = 0.05) by twofold or greater (full list of genes is in National Center for Biotechnology Information [NCBI] Gene Expression Omnibus [GEO] series accession no. GSE23793). Pathway enrichment analysis using MetaCore (pathway analysis software from GeneGo)
compared with that of F4/80 FoxP3+ cells. There were 663 genes expressed in both cell types (full list of genes is in GEO series accession no. GSE23793). The two salient pathways that these cells have in common are signaling through cyclic AMP (P = 8 × 10^{-5}) and signaling through the TGF-βR family (P = 1 × 10^{-4}). This analysis illustrates that the presence of Foxp3 in two different immune cell types results in some concordance, as well as in significant differences in gene expression. The differences in basal gene expression between F4/80 FoxP3+ and F4/80 FoxP3- cells are an indication that these cells have different biological functions that affect the outcome of the immune response. The data discussed in this publication has been deposited in NCBI GEO and are accessible through GEO series accession no. GSE23793 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23793).

Evaluation of cytokines, chemokines, and other factors produced by CD11b+F4/80+Foxp3- and CD11b+F4/80+Foxp3+ cells

M1-MØs are characterized by the production of NO and are cytotoxic, whereas M2-MØs produce Arg, which facilitates
...conversion of Foxp3+ MO is dose dependent and the conversion of large numbers of F4/80+Foxp3+ cells requires high concentrations of TGF-β (Fig. 5A). Overall, these results further confirm that F4/80+Foxp3− and F4/80+Foxp3+ cells have significant differences in cytokine, chemokine, and growth factor profiles, suggesting that these cells play different roles within the immune system.

Role of CD11b+ F4/80+Foxp3+ cells in tumor promotion

We analyzed whether the F4/80+ Foxp3+ cells are found within a B16 melanoma tumor model and whether this population is distinct from the MDSC (CD11b+Gr-1+) population. As expected, a large population of CD11b+ cells was found within the tumor. Of the CD11b+ population, only ~2% of the cells were F4/80+Foxp3+ and ~10% were F4/80+, whereas the majority of the CD11b+ cells were GR-1+ (Fig. 10A). These results demonstrate that different CD11b+ subpopulations can be found within a tumor and that they might serve different functions for tumor promotion or progression. We were surprised that only a small fraction of the CD11b+ cells were Foxp3+ within the tumor. We have previously demonstrated that T reg cells accumulate in this tumor over time (Sharma et al., 2008b). Therefore, we investigated whether F4/80+Foxp3+ cells accumulate within the tumor over time. Surprisingly, we observed that at earlier stages, when tumor volume is low (day 10), higher numbers of F4/80+Foxp3+ cells are present within the tumor compared with the levels of CD4+Foxp3+ cells (Fig. 10B). At later stages, when the tumor load is larger (30 d), the numbers of F4/80+Foxp3+ cells were lower compared with the levels of CD4+Foxp3+ cells (Fig. 10B). These results suggest that F4/80+Foxp3+ cells might be critical at earlier stages of tumorigenesis or during tumor initiation. Several studies have indicated that MDSC and DCs are able to induce the conversion of T reg cells (Huang et al., 2006; Yamazaki et al., 2008),...
We examined whether F4/80^+Foxp3^+ cells induce de novo conversion of T reg cells. To avoid the possibility that contaminating CD4^+Foxp3^+ cells were present in the cultures and expanded by the presence of F4/80^+Foxp3^+ cells, we used the Foxp3–Diphtheria toxin (DT) receptor (DTR) mice for these experiments. Foxp3-DTR mice were treated with 50 µg/kg DT (Johanns et al., 2010). Under these conditions, >99% of the T reg cells were depleted in the periphery and spleen (Fig. 10 C, inset). CD4^+Foxp3^− T cells were sorted from DT-treated Foxp3-DTR mice and co-cultured in the presence of double-sorted F4/80^+Foxp3^+ or F4/80^+Foxp3^− cells. Our results indicate that co-culture of F4/80^+Foxp3^+ cells and CD4^+Foxp3^− cells resulted in de novo conversion of T reg cells (Fig. 10 C), whereas no conversion was observed in the presence of F4/80^+Foxp3^- cells (Fig. 10 C). Based on these results, we wanted to establish whether F4/80^+Foxp3^+ cells are involved in promoting tumor growth. Lin et al. (2005) demonstrated that F4/80 KO mice have normal development and distribution of MØ; however, in these animals it is not possible to induce peripheral tolerance. Because the lack of F4/80 had been implicated in peripheral tolerance, we speculated that perhaps the rate of tumor growth might be different between F4/80 KO and wild-type mice. There are no previous studies indicating whether or not F4/80 KO mice support tumor growth. This was tested by inoculating 10^6 B16 tumors into F4/80 KO mice. Our results showed that F4/80 animals did not develop tumors (Fig. 10 D). We do not fully understand why F4/80 KO mice do not support tumor growth. It is possible to speculate that the tolerogenic function of MØ is altered in these animals, that T reg cells are not induced in these animals, as indicated by Lin et al. (2005), or, because F4/80 is a molecule involved in adhesion, it might be important for cell–cell interaction function or migration. We took advantage of the F4/80 KO mice to investigate whether F4/80^+Foxp3^+ cells have a role in promoting tumor growth. For these experiments, B16 tumor cells were mixed at a 1:1 ratio with single-sorted F4/80^+Foxp3^+ or F4/80^+Foxp3^- cells and implanted into F4/80 KO mice. As shown in Fig. 10 D, in the presence of F4/80^+Foxp3^+ cells, the tumor growth was restored almost to the same rate as the C57BL/6 mice. A mixture of B16 cells and F4/80^+Foxp3^- cells minimally promoted the tumor growth. To further evaluate whether F4/80^+Foxp3^- cells are critical in supporting and promoting tumor growth, we tested a lower B16 cell to F4/80^+Foxp3^- cell ratio (1:0.1). At this lower ratio, F4/80^+Foxp3^- cells were superior than F4/80^+Foxp3^- cells at a 1:1 ratio in promoting tumor growth (Fig. 10 D), indicating the importance of Foxp3^+ MØ in tumor promotion. Similarly, Ehirchiou et al. (2007) also found that in CD11b KO mice peripheral tolerance cannot be induced in these animals. We analyzed whether CD11b KO mice support the tumor growth of B16 tumors. Our results demonstrated that similar to the F4/80 KO mice, these animals also did not develop tumors (Fig. S5). When B16 cells were mixed with F4/80^+Foxp3^- cells, tumors grew in CD11b KO mice but not with F4/80^+Foxp3^- cells (Fig. S5). It can be argued that F4/80^+Foxp3^- cells are relevant in the F4/80 KO or CD11b KO mice because these animals might have a defect in the MO compartment. Therefore, we wanted to evaluate the role of these cells in tumor development in normal mice. For this aim, we identified the minimum number of B16 cells that do not form tumor in C57BL/6 mice (1 × 10^5). Then, 1 × 10^5 B16 tumor cells were mixed at 1:1 or 1:0.1 ratios with F4/80^+Foxp3+ or F4/80^+Foxp3^- cells and implanted into C57BL/6 mice. As shown in Fig. 10 E, F4/80^+Foxp3+ cells at a 1:1 or a 1:0.1 ratio rescued the formation of tumors, whereas F4/80^+Foxp3^- cells did not. These results further support the notion that one of the roles of F4/80^+Foxp3+ cells is to promote tumor growth.

**DISCUSSION**

For the first time, we report a unique population of CD11b^+ F4/80^+ (and CD68^+) cells that express Foxp3, suggesting that these cells are of MO origin. Our results are in agreement with the findings from Zuo et al. (2007) indicating that Foxp3 has a broad function outside of T reg cells in which Foxp3 could be expressed in epithelial cells of multiple organs (Chen et al., 2008) or, as indicated from our studies, in other immune compartments.
Figure 10. CD11b+F4/80+Foxp3+ cells promote tumor growth. (A) A 30-d-old B16 tumor was stained with anti–CD11b-APC, anti–Foxp3-FITC, and anti–F4/80-PE or anti–GR1-PE mAb. CD11b+ cells were gated and percentages of CD11b+Foxp3+ cells were determined. Percentages of double-positive CD11b+F4/80 or CD11b/GR-1 cell were determined from the gated CD11b+F4/80+ cells and CD11b+/Foxp3− cells. Data shown are from a single experiment that is representative of at least three independent experiments. (B) C57BL/6 mice were inoculated with 1 × 106 B16 cells and animals were sacrificed on day 10, 20, or 30. The absolute numbers of CD11b+F4/80+Foxp3+ and CD4+Foxp3+ cells within the tumor was evaluated. Five animals were included per group. Data are representative of two experiments. (C) Foxp3-DTR mice were treated with 50 mg/kg DT for 2 d. On day 3, animals were sacrificed. With this protocol, >99% of the T reg cells were depleted in spleen (inset). After treatment, CD4+Foxp3− cells from Foxp3-DTR mice were sorted and plated on plates coated with anti-CD3 antibody and anti-CD28 antibody and co-cultured in the presence of double-sorted F4/80+Foxp3− or F4/80+Foxp3+ cells at different CD4+Foxp3− cell to F4/80+Foxp3−/+ cell ratios. After 3 d of incubation, percentages of converted CD4+Foxp3+ were analyzed. One of at least three independent experiments is shown. (D) C57BL/6 and F4/80 KO mice were implanted with 1 × 106 B16 cells. Single-sorted CD11b+F4/80+Foxp3+ cells from Foxp3-GFP mice (106 or 105) were mixed with B16 cells (106) and implanted s.c. into F4/80 KO mice and tumor growth was analyzed. In the control F4/80 KO group, mice were implanted with a mixture of 106 B16 cells and 106 single-sorted CD11b+F4/80+Foxp3− cells and tumor growth was evaluated. Five animals were included per group. Data are representative of two experiments. (E) 105 single-sorted F4/80+Foxp3+ or 105 single-sorted F4/80+Foxp3+ cells from Foxp3-GFP mice, mixed with 106 B16 cells, were implanted s.c. into C57BL/6 mice and tumor growth was analyzed. As a control for tumor growth, C57BL/6 mice were implanted with 3 × 105 B16 cells. Five animals were included per group. Data are representative of two experiments. Error bars represent SE.
cells besides T cells. This newly identified population of Foxp3+ MO is distributed in all major lymphoid organs. The presence of Foxp3+ MO in RAG-1 KO mice that lack T cells confirms that this population of Foxp3-positive cells is not of T cell origin. Additionally, morphological and phagocytic assays confirmed that F4/80 Foxp3+ cells have the characteristics and function of MO. A major function of T reg cells is their ability to inhibit other cells. Our results showed that only F4/80 Foxp3+ cells, and not F4/80 Foxp3− cells, were able to inhibit the proliferation of T cells to the same degree as CD4+Foxp3+ T reg cells. The suppressive capabilities of F4/80 Foxp3+ cells did not depend on the presence of T reg cells. The expression of Foxp3 is critical to confer suppressive capabilities to F4/80 Foxp3+ cells because depletion of Foxp3 mRNA results in the inability of these cells to suppress T cell proliferation. Phenotypic characterization of F4/80 Foxp3+ cells revealed that these cells express lower levels of CD11b and higher levels of F4/80 compared with F4/80 Foxp3− cells. It is not yet clear why the Foxp3+ cells express CD11b and F4/80 differentially. Several studies have demonstrated that the level of F4/80 expression on MO is associated with tolerogenic effector function (van den Berg and Kraal, 2005). Lin et al. (2005) demonstrated that the F4/80 molecule is involved in the induction of immunological tolerance. Perhaps higher expression of F4/80 is associated with suppressive activity, as reported by Sica and Bronte (2007) and Kusmartsev and Gabrilovich (2005), where intratumoral Gr-1+ MDSC could mature into GR-1− F4/80+ cells with immunosuppressive properties. Our results show that CTLA-4, GITR, and IL4R are differentially expressed between Foxp3+ and Foxp3− MO. Regardless of the expression of these cellular markers, F4/80 Foxp3+ cells have suppressive capabilities because depletion of Foxp3 eliminated the inhibitory capacity of these cells. Although it has been demonstrated that MDSC-expressing IL4Rs are suppressive, it has also been demonstrated that MDSCs from IL4R KO mice are suppressive as well. Additionally, T reg cells can also have different levels of CTLA-4 and GITR expression, and all CD4+Foxp3+ T cells are still suppressive; hence, these results are compatible with ours. We still do not fully understand the biological role of CTLA-4, GITR, and IL4R expression in this subpopulation of MO; however, the expression of these cellular markers might be related with a state of activation/maturation or these markers could be important in migration or other functions.

In contrast with other APCs with immune-regulatory properties that are induced under polarizing conditions such as infection or neoplastic diseases, a major characteristic of F4/80 Foxp3+ cells is that they are a natural occurring population with suppressive capabilities. The main mechanism by which F4/80 Foxp3+ cells inhibit T cells is through secreted soluble factors because in Transwell assays, these cells could still suppress the proliferation of T cells. PGE2 is the main soluble factor used by F4/80 Foxp3+ cells for suppression. This is in agreement with previous studies reporting that monocytes (Bryn et al., 2008), other APCs (Yang et al., 2003), or T reg cells (Mahic et al., 2006) can suppress T cell function through the secretion of PGE2. These results show that PGE2 is a common mechanism used by the immune system to maintain homeostatic balance of the immune responses. There is strong evidence that T reg cells could inhibit through contact-dependent mechanisms by inducing apoptosis of T cells (Pandiyan et al., 2007). Our microarray data points to differences in the expression of genes that are involved in apoptosis induction of target cells. We examined the expression of several of these markers by FACS analysis. Our data indentified multiple molecules, such as TRAIL, CD200r, Lag3, B7-H1, B7-H4, and PD1, that are highly expressed in Foxp3+ MO compared with Foxp3− MO (unpublished data). Therefore, it could be hypothesized that Foxp3+ MO might regulate immune responses through the induction of cell death (under evaluation).

Our results also demonstrate that F4/80 Foxp3− cells could be converted to express Foxp3, and these cells become phenotypically similar to natural F4/80 Foxp3+ cells with the capability to inhibit T cells. These cells can be converted using growth factors such as TGF-β, VEGF, or toll-like receptor ligands (TLR-L). This could have important clinical ramifications because tumors secreting TGF-β or VEGF may induce MO to express Foxp3, potentially leading to the generation of an immunosuppressive environment and the promotion of tumor growth. This may apply also to infectious diseases where the presence of TLR-L might induce F4/80 Foxp3+ cells helping to control the immune responses. Future studies will determine the importance and biological role of induced F4/80 Foxp3+ cells in regulating immune responses. Overall, our results indicate that natural and induced F4/80 Foxp3+ cells have indistinguishable immune-regulatory properties.

Based on the transcriptome analysis, it is clear that Foxp3+ and Foxp3− MO are distinct subpopulations of MO that play different biological roles in the immune system. We observed that many genes involved in immune regulation are differentially expressed between Foxp3+ and Foxp3− MO. The high expression in Foxp3+ MO of genes, such as Foxp3, IL-4R, Arg2, various phospholipases (Pla2g family), and others, correlate strongly with immune-suppressive activity. Genes involved in immune regulation, such as MAF and IGF1 which could drive a Th2 response and are capable of influencing the immune responses (Hunt and Eardley, 1986; Voice et al., 2004; Yamane et al., 2005), are highly up-regulated in Foxp3+ MO. Genes involved in prostaglandin production (e.g., PTGS1 [cyclooxygenase-1], Pla2g4c, Plag5, and Pla2g6) are also up-regulated in Foxp3+ MO, which correlates with the mechanism of inhibition used by these cells. Foxp3+ MOs also express a variety of growth factor genes (Fig. 8). It is possible that this palette of growth factors may contribute to the accelerated tumor growth and increased tumor volume seen in our in vivo analyses. One unexpected finding is that Foxp3+ MOs exhibit robust up-regulation of complement components (most by 60-fold; Fig. 8) and CD55 and CD59. These data strongly correlate with a recent study indicating that complement promotes tumor growth by regulating MDSC (Markiewski et al., 2008).
Because the Foxp3 transcription factor defines mouse T reg cells and is also highly expressed in this new regulatory MO population, we investigated whether there is transcriptional homology between these two regulatory cell populations. Our data indicates that many T reg cell–specific genes were also differentially expressed in Foxp3+ MO. It has been suggested that mRNA expression of heme oxygenase 1 (Hmox1 encoding HO-1) is linked to the induction of Foxp3 in CD4+CD25+ T reg cells (El Andaloussi and Lesniak et al., 2007). Our microarray data indicates that Hmox1 is >30-fold higher in Foxp3+ MO than in Foxp3− MO. Deaglio et al. (2007) demonstrated that coexpression of CD39 and CD73 provide suppressive capabilities to T reg cells. The microarray data indicate that CD39 (Nt5e) and CD73 (Entpd1) are expressed more than sixfold higher in Foxp3+ MO than in Foxp3− MO but similar to levels in T reg cells. These are examples indicating some transcriptional concordance between T reg cells and Foxp3+ MO, confirming the link between Foxp3 expression and suppressive capacity. Further studies will be necessary to unravel the precise role of genes that are differentially regulated in F4/80+Foxp3− and F4/80+Foxp3+ populations and how they relate to the function of Foxp3+ MO.

Based on the type of cytokines and other factors secreted by F4/80+Foxp3+ cells, we believe that these cells are associated with suppression, induction of immune-suppressive networks, and tumor promotion. F4/80+Foxp3+ cells produce more Arg-2, IL-10, CXCL4, CCL7, CCL9, CXCL12, CXCL13, Pdgf, and VEGF. Arginase promotes tumor growth and progression (Bronte and Zanovello, 2005). Secretion of IL-10a usually correlates with tumor invasiveness and a poor prognosis (Castrilli et al., 1997). CXCL4 down-regulates IFN-γ production and promotes the secretion of Th2 cytokines (Romagnani et al., 2005). CXCL12 and CXCL13 play a key role in the trafficking of hematopoietic cells but are also involved in tumor growth and metastasis (Müller et al., 2001). CXCL9 can promote tumor escape and facilitates tumor metastases (Amatschek et al., 2011). CCL7 and CCL9 are chemoattractants responsible for recruiting MDSC (Kitamura et al., 2007; Sawanobori et al., 2008). VEGF is a major factor that promotes tumor angiogenesis. Additionally, VEGF could increase the number of immature DCs and accumulation of MDSC (Gabrilovich and Nagaraj, 2009) at the tumor site. Pdgf-BB is overexpressed in various tumors such as pancreas, breast, ovarian, and others (Markiewski et al., 2008), and it is thought that Pdgf-BB is involved in tumor formation by promoting angiogenesis and recruiting and activating stromal cells (Markiewski et al., 2008). This indicates that F4/80+Foxp3+ cells could use multiple mechanisms to promote tumor growth: (1) secreting growth and suppressive factors; (2) promoting the conversion of T reg cells; (3) inducing networks of immune suppression; and (4) directly inhibiting effector cells. Our data indicate that higher numbers of F4/80+Foxp3+ cells are found at early stages during the progression of tumor compared with T reg cells. Importantly, F4/80+Foxp3+ cells restored the growth of B16 tumors even at low tumor cell/Foxp3+ MO ratios in F4/80 KO and CD11b KO mice. In normal mice, when animals were implanted with low numbers of tumor cells they were not able to form tumors; however, when these low numbers of tumor cells were mixed with F4/80+Foxp3+ cells at 1:1 or 1:0.1 tumor cell/Foxp3+ MO ratios tumors developed. Collectively, these results suggest that F4/80+Foxp3+ cells might be critical at the initial step of tumor formation where they create an environment that favors tumor growth. Most likely, F4/80+Foxp3+ cells promote tumor growth by concurrently using the mechanisms described in this section. These studies demonstrated for the first time that the presence of F4/80+Foxp3+ cells is very important for tumor initiation and promotion. Perhaps immunotherapeutic strategies directed to eliminate or block the function of these cells could be critical to impair tumor growth and prevent metastatic lesions.

Whether or not F4/80+Foxp3+ cells are more related to M2-MO and F4/80+Foxp3− cells to M1-MO needs to be defined (Martinez et al., 2008). Based on the cytokine assays and transcriptional profile, F4/80+Foxp3+ cells have some characteristics of M2-MO, whereas F4/80+Foxp3− cells resemble M1-MO. It is important to emphasize that the distinction of M1 and M2 MO is based on the type of stimuli to polarize these cells, whereas the F4/80+Foxp3+ cells identified in these studies are a naturally occurring population with immune-regulatory properties. Based on our in vitro and in vivo functional analyses, it is clear that F4/80+Foxp3+ and F4/80+Foxp3− populations have different biological functions.

In summary, these studies describe and characterize for the first time a distinct subpopulation of CD11b+F4/80+ MO that expresses Foxp3. The expression of Foxp3 confers to these MOs the ability to inhibit T cells. Based on the immunoregulatory properties of CD11b+F4/80+Foxp3+ cells, we termed these cells Foxp3+ regulatory macrophages. Presently, we do not completely understand the biology of Foxp3+ regulatory macrophages and much remains to be elucidated about the pathological functions of these cells. Further studies will determine the role of Foxp3+ regulatory macrophages in tolerance, autoimmunity, tumor immunity, organ transplantation, allergy, and microbial immunity and how these cells can help in the maintenance of the homeostatic balance of the immune system.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from Harlan. RAG-1 KO mice were purchased from The Jackson Laboratory. The Foxp3-GFP knockin (Fontenot et al., 2005) and Foxp3-DTR (Kim et al., 2007) mice were obtained from A. Rudensky (Sloan Kettering, New York, NY). The F4/80 KO mice (Lin et al., 2005) were obtained from J. Stein-Streilein (Schepps Eye Research Institute, Boston, MA). Mice were maintained under specific pathogen-free conditions in our animal facility, and experiments were performed under the approval of Institutional Animal Care and Use Committee of the Mayo Clinic.

Isolation of CD11b+F4/80+Foxp3+ cells. Organ samples from C57BL/6 mice were stained with anti-CD11b-APC and anti-F4/80-PE mAb (eBioscience) and intracellularly stained with anti–Foxp3-FITC (eBioscience). To isolate CD11b+F4/80+Foxp3+ cells from Foxp3-GFP mice, spleen or bone marrow cells were treated with biotin-labeled anti-CD2, anti-CD3, and...
anti-CD45R mAbs (BD) and then incubated with anti-biotin microbeads (Invitrogen) to deplete these populations. Samples were then double-stained with anti-CD11b-APC and anti-F4/80-PE mAbs. The CD11b+ F4/80- Foxp3+ and CD11b+ F4/80+Foxp3+ cell fractions were collected using three-color sorting on a FACSAria (BD). In some experiments, CD11b+ F4/80-Foxp3+ and CD11b+ F4/80+Foxp3+ cells were sorted twice to enrich the populations and avoid contaminations from other populations. At least 10 Foxp3-GFP mice were used per experiment to isolate sufficient numbers of CD11b+ F4/80+Foxp3+ cells.

**Phagocytosis assays.** 10 single-sorted CD11b+ F4/80+Foxp3+ and CD11b+ F4/80- Foxp3+ cells from bone marrow were incubated with Alexa Fluor 700 beads at 1:10, 1:25, or 1:50 cell/bead ratios for 4 or 24 h. At the determined times, cells were evaluated for the incorporation of beads by flow cytometry.

**Suppression assay.** CD4+ T cells from spleen were enriched (>95-98% purity) by magnetic purification (Invitrogen) and plated at 10^5 cells/well alone or co-cultured with bone marrow-derived single- or double-sorted CD11b+ F4/80+Foxp3+ or CD11b+ F4/80+Foxp3+ cells (1 × 10^5, 5 × 10^4, or 2.5 × 10^4 cells/well) in a 96-well flat-bottom plate coated with 2 µg/ml anti-CD3 antibody and 2 µg/ml anti-CD28 antibody. Proliferation was measured by [H]-Thymidine incorporation after 72 h of incubation.

**Transwell assay.** Transwell experiments were performed in 96-well plates with pore size 0.4 µm (Millipore). 1 × 10^5, 5 × 10^4, or 2.5 × 10^4 single-sorted CD11b+ F4/80+Foxp3+ cells were added in the upper chamber. 1 × 10^5 freshly purified CD4+ T cells were cultured in the bottom chamber and stimulated with 2 µg/ml anti-CD3/anti-CD28 plus irradiated APCs. CD4+ T cells were plated in the bottom chamber without adding CD11b+ F4/80+Foxp3+ cells in the upper as a control of proliferation. After 72 h in culture, top chambers were removed and [H]-Thymidine was added directly to the responder CD4+ T cells in the bottom chambers of the original Transwell plate for the final 16 h of the assay.

**FOXP3 gene silencing with siRNA treatment.** Foxp3 siRNA and FITC-labeled control siRNA (Santa Cruz Biotechnology) was prepared as specified by the manufacturer. Using single-sorted F4/80+Foxp3+ and F4/80+Foxp3- cells from BM, transient transfections were performed using Lipofectamine 2000 (Invitrogen) at a concentration of 20 nM and verified by flow cytometry to visualize the FITC control siRNA. Cells were cultured for 48 h, at which time maximum silencing was observed. Then, the cells were used for quantitative RT-PCR and proliferation assay.

**Quantitative RT-PCR verification of gene expression knockdown.** Total RNA was isolated from single-sorted CD11b+ F4/80+Foxp3+, CD11b+ F4/80- Foxp3+ (bone marrow), and T reg (splen) cells with the RNeasy mini kit (QiAGEN). Reverse transcription was done using SuperScript III, and real-time PCR used Power SYBR Green PCR Master Mix (Applied Biosystems) on the ABI7000 platform. FOXP3 primers (foxp3 forward, 5'-TTGCCAGGCGCATCTT-3'; foxp3 reverse, 5'-TGCCCT-CCTCAGAGAGAAGTG-3') and GAPDH primers (GAPDH forward, 5'-ACCCGAAGACTGTGGATGG-3'; GAPDH reverse, 5'-CAGAT-TGGGGTAGAACAC-3') were used to determine FOXP3 mRNA levels in each population.

**PGE2 production.** Analysis of PGE2 production was performed using an ELISA kit and protocol developed by Cayman Chemical. In brief, single-sorted CD11b+ F4/80+Foxp3+ or CD11b+ F4/80+Foxp3- cells (10^5 cells/well) from bone marrow were cultured for 48 h in complete media. Cell culture supernatants were collected, filtered, and assayed for presence of PGE2.

**Phenotypic characterization of CD11b+ F4/80+Foxp3+ cells.** Bone marrow–derived double-sorted CD11b+ F4/80+Foxp3+/+ cells from Foxp3-GFP mice were stained with anti-GR-1-Biotin, anti-CTLA-4-Biotin, anti-GITR-Biotin, and anti-IL-4R-Biotin and then incubated with Streptavidin–Alexa Fluor 700 (Bioscience) and analyzed by flow cytometry. To evaluate, arginase and iNOS antibodies were purchased from BD.

**Conversion analysis.** 1 × 10^4 bone marrow–derived double-sorted CD11b+ F4/80+Foxp3- cells from Foxp3-GFP mice were cultured in 24-well plates in complete medium in the presence or absence of 1 µg/ml LPS, 1 µg/ml CpG, 5 ng/ml TGF-β, or 5 ng/ml VEGF. After 3 d of incubation, cells were harvested and stained with anti-CD11b-APC mAb and anti-F4/80-PE mAb. The induction of Foxp3(GFP) expression was analyzed by flow cytometry.

**Multiplex assay.** Bone marrow–single-sorted CD11b+ F4/80+Foxp3+/+ populations were cultured in complete medium for 48 h and supernatants collected. The levels of cytokines and chemokines were assayed using multiplex luminVent beads (Invitrogen) as described previously (Sharma et al., 2008a). The lower limit of detection was 1.5 pg/ml for each cytokine or chemokine.

**Expression microarray analysis.** RNA was isolated from CD11b+ F4/80+Foxp3+ as well as from CD4+ Foxp3+ populations from three independently double-sorted cell harvests, using RNeasy min columns (QiAGEN), according to the manufacturer’s instructions. RNA was measured with the NanoDrop 1000 (Thermo Fisher Scientific) and its integrity verified with the BioAnalyzer 2100 (Agilent Technologies). 500 ng RNA per sample was labeled using the low-input linear amplification kit, one color (Agilent Technologies). Dye incorporation and amplified RNA yield were measured with the NanoDrop. 1.6 µg of each cy3-labeled sample was hybridized to a 4X44K mouse whole genome slide (Agilent Technologies), washed, and feature extracted according to the manufacturer’s (Agilent Technologies) instructions for single channel experiments. All experiments passed quality control metrics with a mean correlation coefficient between replicates of 0.97 for CD11b+ F4/80+Foxp3+ arrays, 0.94 for CD11b+ F4/80+ Foxp3- arrays, 0.99 for CD4+ Foxp3+ arrays, and 0.92 for CD4+ Foxp3- arrays. Data analysis was done using GeneSpring GX11 software (Agilent Technologies), using default data normalization settings for single channel Agilent arrays. In brief, for each matched pair, low expressers and spots flagged as absent were removed in three of the six samples, followed by a Student’s t test (with a cutoff of P < 0.05) to retain only genes that are statistically differentially expressed, and finally a twofold change minimum between the two populations was applied to retain genes with significant changes in gene expression. The biological replicates were averaged and differential gene expression was expressed in log2. The data discussed in this publication has been deposited in National Center for Biotechnology Information’s Gene Expression Omnibus, and is accessible through GEO series accession no. GSE32793 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32793).

**Tumor growth analysis.** C57BL/6 mice were implanted with 10^5 B16 cells and animals were sacrificed on days 10, 20, and 30, and the levels of CD11b+ F4/80+Foxp3+ and CD4+ Foxp3+ cells were analyzed. 10^5 or 10^4 single-sorted CD11b+ F4/80+Foxp3+/+ cells were mixed with 10^6 B16 cells and implanted i.c. into F4/80 KO mice and tumor growth was analyzed.

**Statistics.** Statistical comparisons between two experimental groups were made with a paired Student’s t test using InStat Software (GraphPad Software). P-values <0.05 were considered significant.

**Online supplemental material.** Fig. S1 shows phenotypic characterization of single-sorted F4/80+Foxp3+ and F4/80+Foxp3- cells from spleens of Foxp3-GFP mice. Fig. S2 demonstrates suppressive function of single-sorted F4/80+Foxp3+ cells against antigen-specific T cells. Fig. S3 shows the conversion of single-sorted F4/80+Foxp3+ cells to Foxp3+ cells from spleen cells and their phenotypic characterization. Fig. S4 shows titration of TGF-β for the induction of Foxp3 MO. Fig. S5 demonstrates that transferring of single-sorted F4/80+Foxp3+ cells rescues tumor growth in CD11b KO mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100730/DC1.
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