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 naive 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.

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Abbreviations used: CTLA-4, cytotoxic T cell–associated antigen 4; DT, diphtheria toxin; DTR, DT receptor; GITR, glucocorticoid-induced TNF receptor family-related gene/protein; iNOS, inducible NO synthase; MDSC, myeloid-derived suppressor cell; PDGF, platelet-derived growth factor; PGE_2, prostaglandin E_2; T reg cell, regulatory T cell; VEGF, vascular endothelial growth factor.

Regulatory T cells (T reg cells) are characterized by the expression of the forkhead lineage-specific transcription factor Foxp3 (Hori et al., 2003). Studies performed in humans and animals indicate that CD4$^+$ T reg cells play an important role in the prevention of autoimmune diseases and inflammation (Dubois et al., 2003; Bacchetta et al., 2006). These cells are critical for the maintenance of peripheral T cell tolerance because their depletion leads to autoimmune disorders (Sakaguchi, 2005; Kim et al., 2007). In addition to T reg cells, a wide range of APCs with regulatory function has been described. The best characterized suppressive cells of myeloid origin are the myeloid-derived suppressor cells (MDSCs; Bronte et al., 2000; Nagaraj and Gabrilovich, 2008). MDSCs are a heterogeneous cell population comprised of macrophages, granulocytes, and DCs at various stages of maturation (Serafini et al., 2006). Similar to T reg cells, MDSCs also display inhibitory properties toward T cells and other immune cells (Apolloni et al., 2000). Increased numbers of MDSCs are associated with neoplastic, inflammatory, and infectious diseases, thereby suppressing the activation and expansion of immune responses (Talmadge, 2007). There are studies indicating that subpopulations of DCs can have regulatory properties capable of inducing peripheral tolerance (Zhang et al., 2004; Chung et al., 2005). For example, Hadeiba et al. (2008) demonstrated that plasmacytoid DCs expressing CCR9 were potent inducers of T reg cell function and

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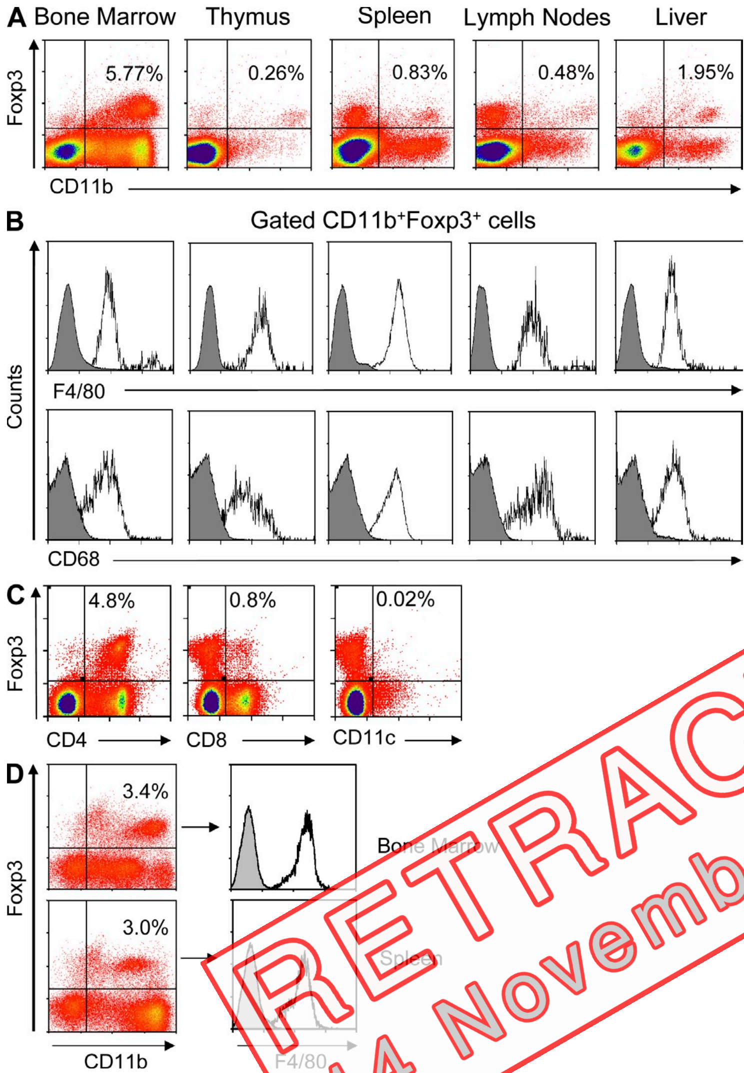

Figure 1. Identification of CD11b$^+$F4/80$^+$Foxp3$^+$ cells. Bone marrow, thymus, spleen, lymph nodes, and liver cells from C57BL/6 mice were stained with anti–CD11b-APC, anti–Foxp3-FITC, anti–F4/80-PE, or anti–CD68-PE. (A) Percentages of double-positive CD11b/Foxp3 cells were determined. (B) CD11b$^+$/Foxp3$^+$-positive cells were gated, and expression of F4/80 and CD68 was determined. (C) Analysis of double-positive CD4/Foxp3, CD8/Foxp3, and CD11c/Foxp3 from spleen cells of C57BL/6 mice. (D) 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.

suppressed antigen-specific immune responses both in vitro and in vivo. Another common subpopulation of immune cells with immune-suppressive functions is macrophages (MØ). Macrophages polarize according to activation stimuli and are classified into M1- and M2-MØ. M1-MØs are tumoricidal and promote tumor immunity, whereas M2-MØ promote angiogenesis, tumor progression, and tissue remodeling and inhibit immune responses (Mills et al., 2000; Martinez et al., 2008). Tumor-associated macrophages are a subpopulation of MØ regarded as critical cells for tumor progression that share many characteristics with M2-MØ (Allavena et al., 2008). Collectively, these data indicate that various subpopulations of suppressive immune cells exist and are critical in regulating the immune responses and maintaining the homeostatic balance of the immune system (Belkaid, 2007; Belkaid and Oldenhove, 2008; Mortellaro et al., 2008; Mantovani et al., 2009). Furthermore, these subpopulations of immune-suppressive cells also play a critical role in autoimmunity, tumor immunity, organ transplantation, or microbial immunity (Belkaid, 2007; Belkaid and Oldenhove, 2008; Mortellaro et al., 2008; Mantovani et al., 2009).

Currently, there are no defined cellular markers that can distinguish activator versus suppressor subpopulations of MØ. Because MØs have regulatory properties that are found only under inflammatory conditions, it is unclear whether MØ 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 MØ (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 F4/80 and CD68 cellular markers. CD11b$^+$Foxp3$^+$ cells were gated (Fig. 1 A, top right quadrant) and the expression of F4/80 and CD68 was analyzed. Our results show that >95% of the CD11b$^+$Foxp3$^+$ cells were positive for F4/80 and CD68 (Fig. 1 B), indicating that these cells are of MØ origin. We confirmed that CD4$^+$ and CD8$^+$ T cell populations express Foxp3; however, CD11c$^+$ populations showed no expression of Foxp3 (Fig. 1 C). CD11b$^+$F4/80$^+$Foxp3$^+$ cells can be observed in bone marrow and spleen from RAG-1 KO mice (Fig. 1 D), confirming that these cells are of MØ origin and not a subpopulation of T cells.

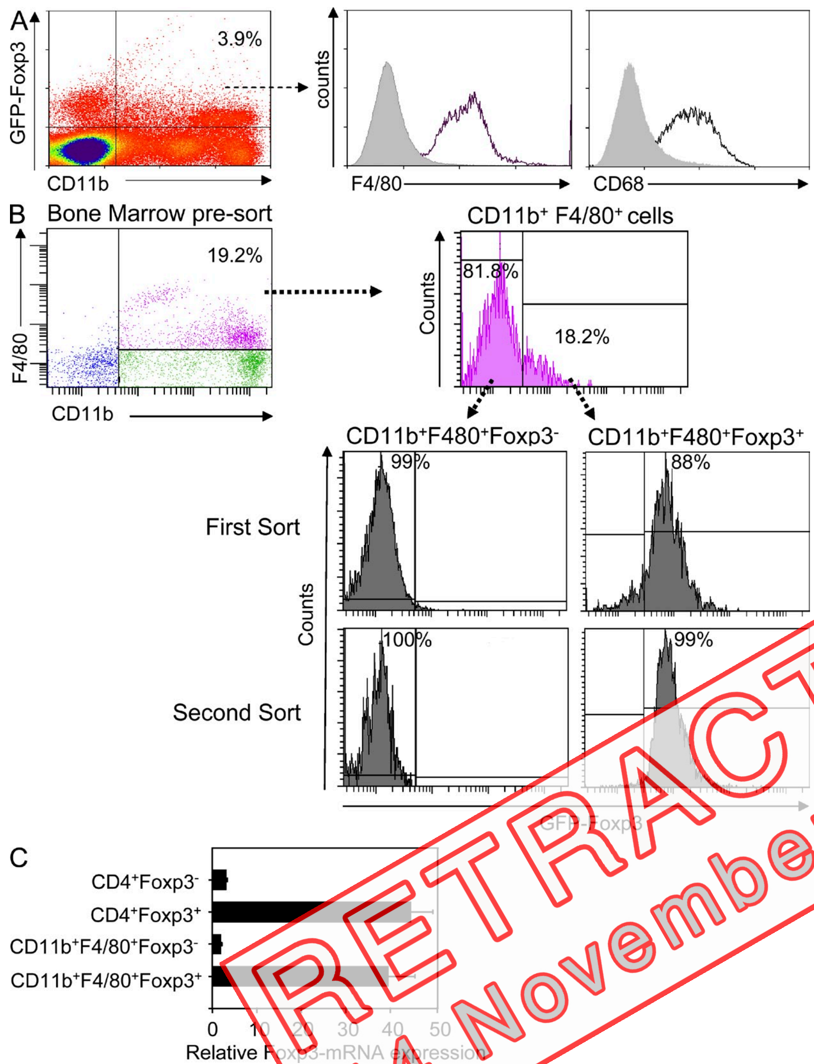

Figure 2. Isolation of CD11b$^+$F4/80$^+$Foxp3$^+$ cells from Foxp3-GFP mice. (A) Percentages of double-positive CD11b/GFP(Foxp3) cells and expression of F4/80 and CD68 were determined from bone marrow cells of Foxp3-GFP mice. 10 Foxp3-GFP mice were used to sort sufficient CD11b$^+$F4/80$^+$Foxp3$^+$ cells. Data represent one experiment of at least 10 separate experiments. (B) Bone marrow cells from Foxp3-GFP mice were stained with anti–CD11b-APC and anti–F4/80-PE. Positive CD11b$^+$/F4/80$^+$ cells were gated and first sorted for GFP(Foxp3)$^+$ (85–90% purity) and GFP(Foxp3)$^-$ (>95% purity) populations. Sorted CD11b$^+$F4/80$^+$Foxp3$^-$ and CD11b$^+$F4/80$^+$Foxp3$^+$ were resorted for a second time to enrich the populations, double-sorted GFP(Foxp3)$^+$ (>98% purity), and double-sorted GFP(Foxp3)$^-$ (100% purity). These levels of purity apply to all the further experiments. Data represent one experiment of at least six separate experiments. (C) Evaluation of Foxp3-mRNA expression from single-sorted F4/80$^+$Foxp3$^-$, F4/80$^+$Foxp3$^+$, CD4$^+$Foxp3$^-$ (>98% purity), and CD4$^+$Foxp3$^+$ (>98% purity) cells. Data represent one experiment of at least five separate experiments. Error bars represent SE.

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 (sorted MØ 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 MØ origin and have MØ 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 MØ 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 MØ origin (Fig. 3 B).

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

After sorting the Foxp3$^+$ and Foxp3$^-$ MØ 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

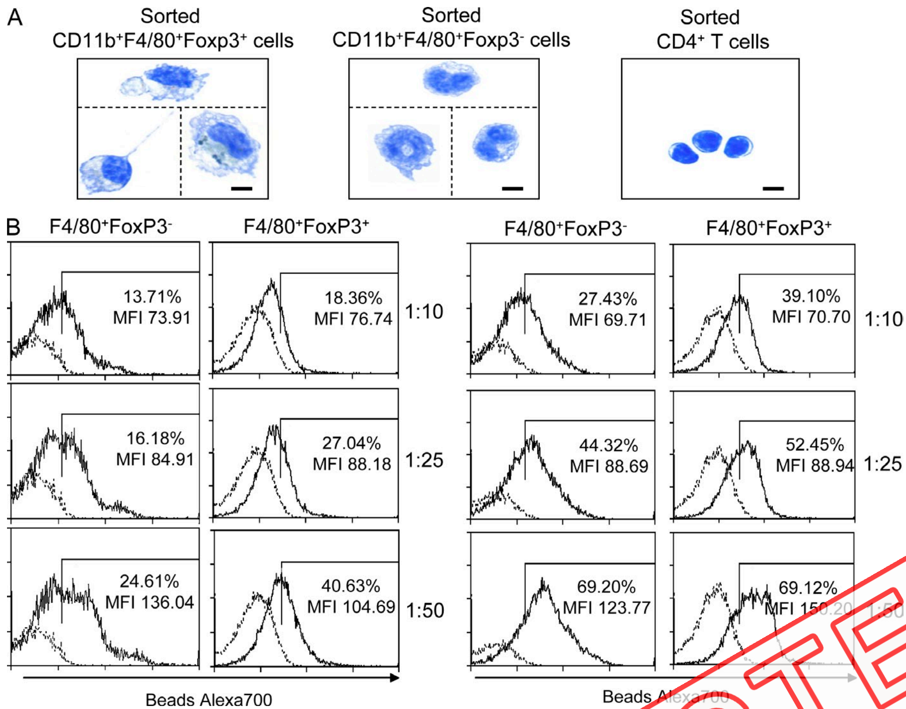

Figure 3. CD11b$^+$F4/80$^+$Foxp3$^+$ cells have characteristics and function of macrophages. (A) Single-sorted F4/80$^+$ Foxp3$^+$ and F4/80$^+$Foxp3$^-$ cells from bone marrow and CD4$^+$ T cells from spleen were stained with Diff-Quick-Fixative for morphological analysis. The dotted lines indicate cells from different fields of the same smear preparation. Bars, 10 µm. Data represent one experiment of at least five separate experiments. (B) 10^5 single-sorted F4/80$^+$Foxp3$^+$ and F4/80$^+$Foxp3$^-$ cells were incubated with Alexa Fluor 700 beads at a 1:10, 1:25, or 1:50 cell/bead ratio for 4 h (left) or 24 h (right). At the determined times, cells were evaluated for the incorporation of beads by flow cytometry. Data represent one experiment of at least three separate experiments.

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 lack suppressive activity, whereas freshly isolated Foxp3$^+$ T reg cells are inhibitory. We investigated whether freshly isolated single- or double-sorted F4/80$^+$ Foxp3$^+$ MØ were naturally inhibitory. As shown in Fig. 5 A, single- or double-sorted F4/80$^+$Foxp3$^+$ cells inhibited proliferation of the CD4$^+$ T cells to the same degree as CD4$^+$Foxp3$^+$ T reg cells. In contrast, no inhibition was observed with F4/80$^+$ Foxp3$^-$ cells. We confirmed that at a lower F4/80$^+$Foxp3$^+$ to CD4$^+$ T cell ratio, the Foxp3$^+$ MØs (single or double sorted) were still suppressive to the same degree as T reg cells (Fig. 5 B), which is compatible with physiological conditions. Because whole CD4$^+$ T cells were used in the proliferation assays, and to rule out that the inhibition was not a result of the presence of T reg cells or that F4/80$^+$Foxp3$^+$ cells enhance the suppressive function of T reg cells, the inhibitory capacity of F4/80$^+$Foxp3$^+$ cells was evaluated in the absence of T reg cells. CD4$^+$Foxp3$^-$ T cells were sorted from Foxp3-GFP mice and co-cultured with single-sorted F4/80$^+$Foxp3$^-$ or F4/80$^+$Foxp3$^+$ cells. The data indicate that F4/80$^+$Foxp3$^+$

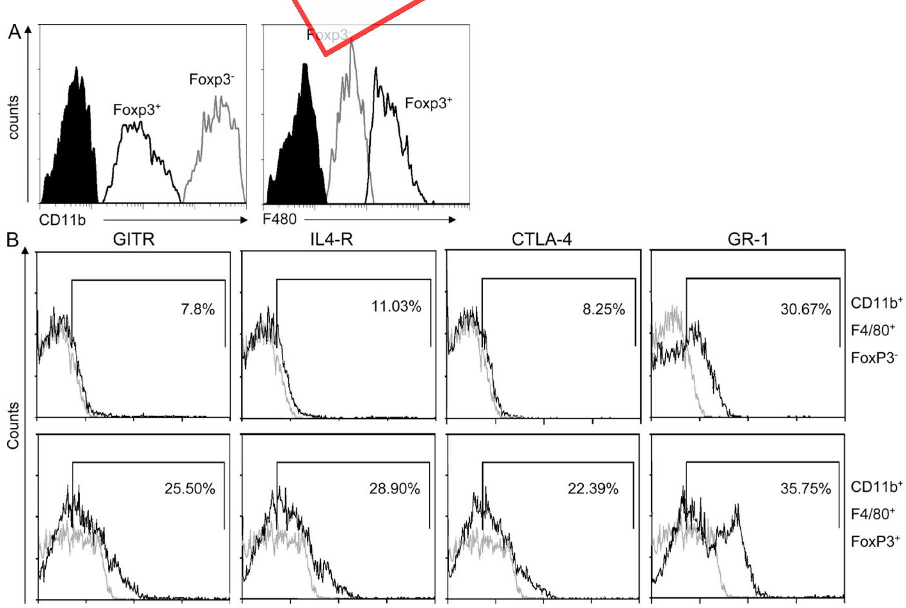

Figure 4. Phenotypic characterization of CD11b$^+$F4/80$^+$Foxp3$^+$ cells from Foxp3-GFP mice. (A) Expression of CD11b and F4/80 was evaluated from bone marrow–derived double-sorted F4/80$^+$Foxp3$^+$ and F4/80$^+$ Foxp3$^-$ cells. Data represent one experiment of at least five separate experiments. (B) Double-sorted CD11b$^+$F4/80$^+$Foxp3$^+$ and CD11b$^+$F4/80$^+$Foxp3$^-$ cells from spleen were stained using anti–GITR-Biotin, anti–IL-4R-Biotin, anti–CTLA-4-Biotin, and anti–GR-1-Biotin mAb plus Streptavidin–Alexa Fluor 700 (black line). Control antibody, gray line. Data represent one experiment of at least five separate experiments.

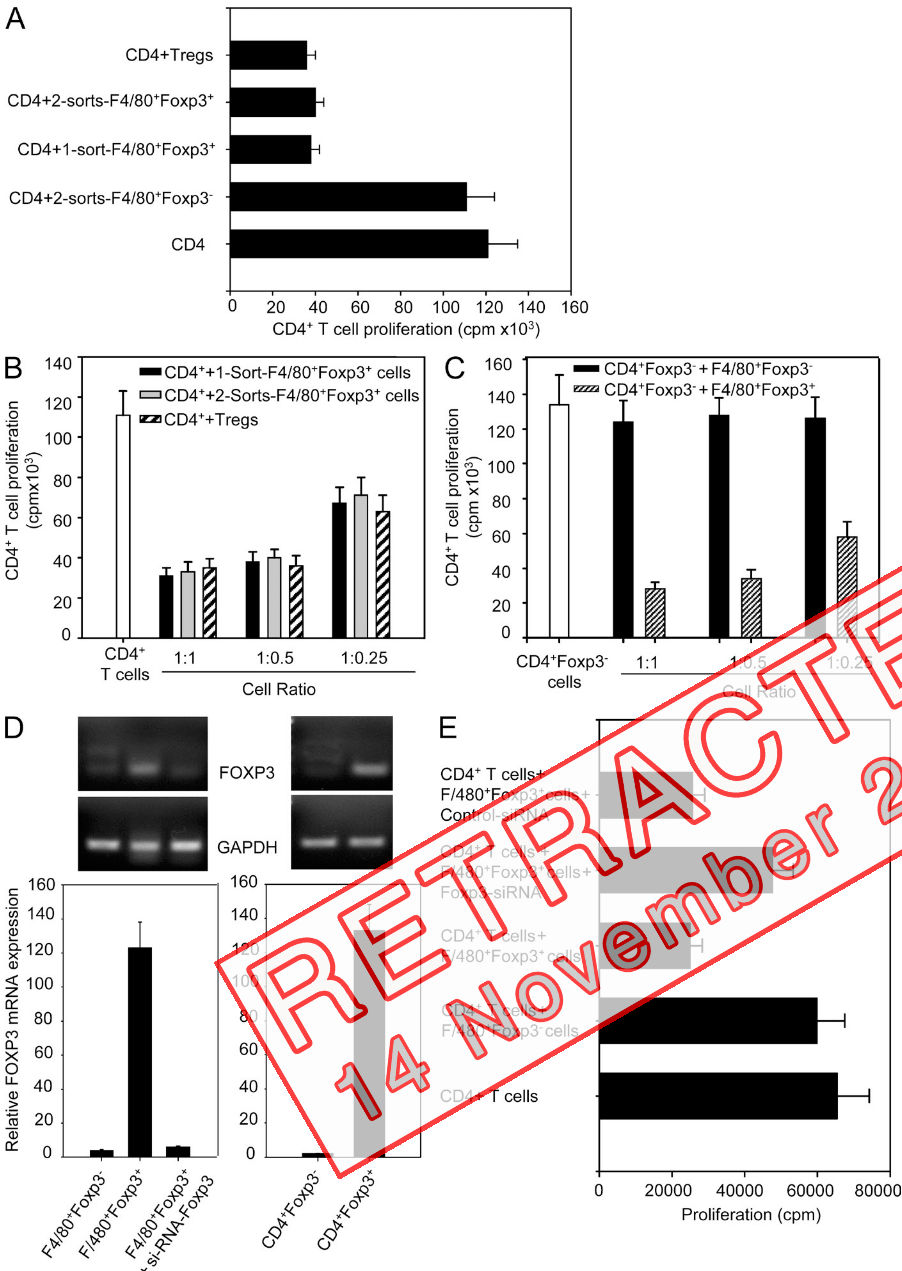

Figure 5. Evaluation of suppressive function of CD11b⁺F4/80⁺Foxp3⁺ cells. (A) To evaluate the suppressive activity of Foxp3⁺ and Foxp3⁻ MØ, enriched CD4⁺ T cells (≥95% purity), and single- or double-sorted F4/80⁺Foxp3⁺ and F4/80⁺Foxp3⁻, cells were co-cultured at a 1:1 ratio and proliferation of CD4⁺ T cells was measured. As a control, purified CD4⁺Foxp3⁺ cells (T reg cells) from Foxp3-GFP mice were mixed at a 1:1 ratio with CD4⁺ T cells. One of at least five independent experiments is shown. (B) To confirm the suppressive capabilities of Foxp3⁺ MØ, CD4⁺ T cells and single- or double-sorted F4/80⁺Foxp3⁺ or CD4⁺Foxp3⁺ cells (T reg cells) were plated at different effector/suppressor ratios and proliferation of CD4⁺ T cells was measured. One of at least five independent experiments is shown. (C) CD4⁺Foxp3⁻ cells (100% purity) were sorted from Foxp3-GFP mice and co-cultured with single-sorted F4/80⁺Foxp3⁺ or F4/80⁺Foxp3⁻ cells at different cell ratios. Proliferation of CD4⁺ T cells was measured. One of three independent experiments is shown. (D) Depletion of Foxp3 from F4/80⁺Foxp3⁺ cells by siRNA. Single-sorted CD11b⁺F4/80⁺Foxp3⁺ cells were transfected with Foxp3-siRNA and mRNA expression of Foxp3 was evaluated. CD4⁺Foxp3⁻ and CD4⁺Foxp3⁺ cells were used as controls for Foxp3 expression. Vertical bars and lanes on the gels correspond to the same conditions. Data represent one experiment of at least three separate experiments. (E) Single-sorted F4/80⁺Foxp3⁺ cells were transfected with Foxp3-siRNA or control siRNA. After 48 h, cells were washed, mixed at a 1:1 ratio with CD4⁺ T cells, and proliferation of CD4⁺ T cells was measured. One of three independent experiments is shown. Error bars represent SE.

cells, but not F4/80⁺Foxp3⁻ cells, inhibited the proliferation of CD4⁺Foxp3⁻ T cells (Fig. 5 C). These results confirm that Foxp3⁺ MØ 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. S2, A 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 MØ (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⁻ MØ were evaluated for their ability to inhibit the proliferation of CD4⁺ T cells. Our results indicate that only supernatants from Foxp3⁺ MØ 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 E_2 (PGE_2). Our results show that only anti-PGE_2 reverses the suppressive effect of the supernatant (Fig. 6 B), indicating that PGE_2 is the inhibitory factor produced by F4/80⁺Foxp3⁺ cells. The levels of PGE_2 secretion from F4/80⁺Foxp3⁻ and F4/80⁺Foxp3⁺ cells were evaluated. Our data indicate that F4/80⁺Foxp3⁺ cells secrete large

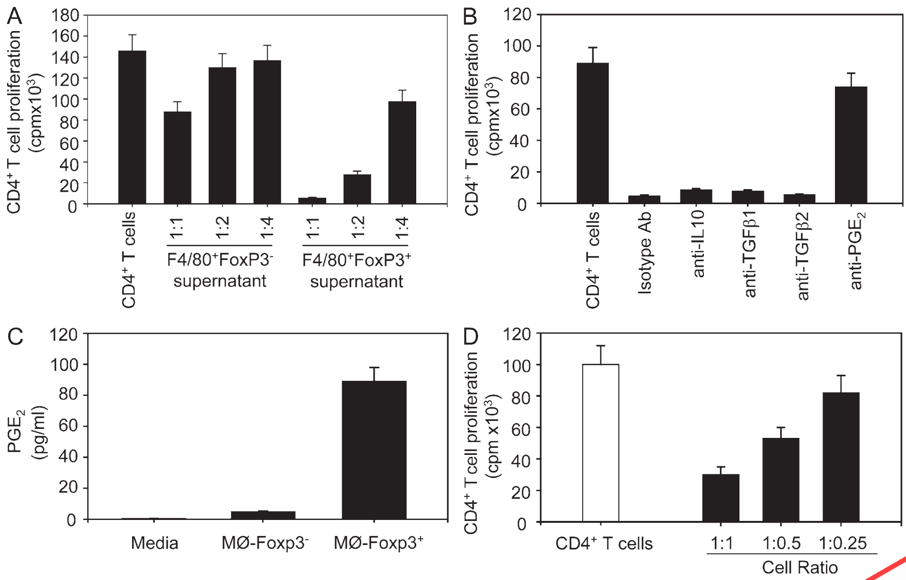

Figure 6. CD11b$^+$F4/80$^+$Foxp3$^+$ cells suppress by secretion of soluble factors. (A) Supernatants from cultured single-sorted F4/80$^+$Foxp3$^+$ and F4/80$^+$Foxp3$^-$ cells were examined at different dilutions for inhibition of CD4$^+$ T cell proliferation. Data represent one experiment of at least five separate experiments. Error bars represent SE. (B) To identify which factor induces the inhibition of T cell proliferation, supernatants from single-sorted F4/80$^+$Foxp3$^+$ cells were incubated with 10 µg/ml anti–IL-10, anti–TGF-β1, anti–TGF-β2, anti-PGE_2 mAb, and isotype control antibody for 2 h before the addition to the cultures. Proliferation of CD4$^+$ T cells was evaluated. Each value represents the mean of triplicate wells ± SE. Proliferation data represent a single experiment of at least three independent experiments. (C) PGE_2 secretion was evaluated from supernatants of single-sorted F4/80$^+$Foxp3$^+$ and F4/80$^+$Foxp3$^-$ cells. Each value represents the mean of triplicate wells ± SE. (D) To assess whether F4/80$^+$Foxp3$^+$ cells induce suppression by cell–cell contact mechanism, transwell assays were performed. Single-sorted F4/80$^+$Foxp3$^+$ cells were added at indicated ratios to the upper chamber, and purified CD4$^+$ T cells were placed in the bottom chamber and stimulated with anti-CD3/anti-CD28 mAb plus irradiated APCs. Proliferation of CD4$^+$ T cells was evaluated. Each value represents the mean of triplicate wells ± SE. Proliferation data are from a single experiment that is representative of at least three independent experiments.

amounts of PGE_2, whereas the secretion of PGE_2 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 in 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. S3). 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)

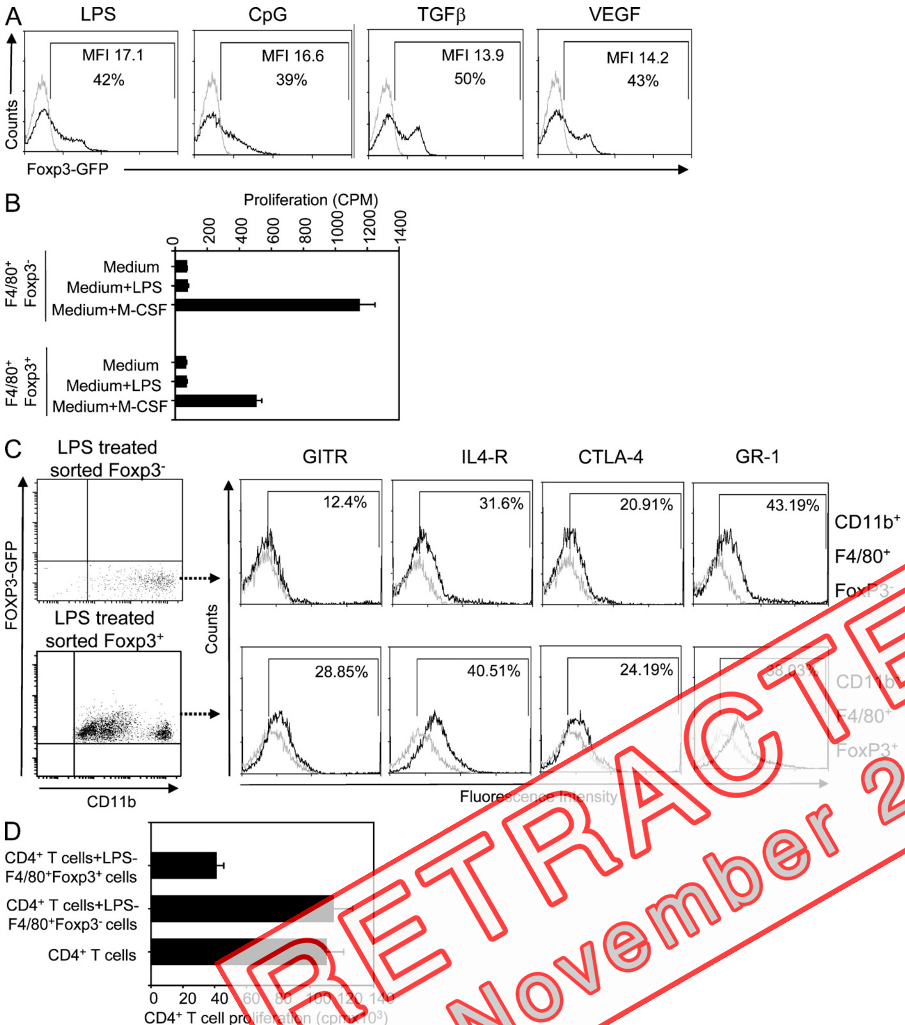

Figure 7. Conversion of CD11b⁺F4/80⁺Foxp3⁻ cells. (A) Bone marrow–derived double-sorted F4/80⁺Foxp3⁻ cells from Foxp3-GFP mice were plated at 5 × 10^5/well in 24-well plates in the presence of 1 µg/ml LPS, 1 µg/ml CpG, 5 ng/ml TGF-β, and 5 ng/ml VEGF. Cells were cultured for 72 h and the expression of GFP (Foxp3) was analyzed. Untreated CD11b⁺F4/80⁺Foxp3⁻ cells, dotted line. Data represent one experiment of at least five separate experiments. (B) Double-sorted F4/80⁺Foxp3⁺ and F4/80⁺Foxp3⁻ cells (10^5 cells/well) were incubated in complete medium alone, medium plus 1 µg/ml LPS, or medium plus 2 ng/ml M-CSF. Cells were incubated for 3 d and proliferation was measured by ^{3}H-Thymidine incorporation. Each value represents the mean of triplicate wells ± SE. Proliferation data are from a single experiment that is representative of at least three independent experiments. (C) Double-sorted F4/80⁺Foxp3⁻ cells from Foxp3-GFP mice were treated with 1 µg/ml LPS. After 72 h of incubation, cells were double-sorted into GFP(Foxp3)⁻ and GFP(Foxp3)⁺ cells. Double-sorted cells were evaluated for the level of expression of GR-1, CTLA-4, GITR, and IL-4 (thin line). Control antibody, dotted line. (D) Double-sorted LPS-F4/80⁺Foxp3⁻ and LPS-F4/80⁺Foxp3⁺ cells were plated at a 1:1 ratio with enriched CD4⁺ T cells and proliferation was measured. Each value represents the mean of triplicate wells ± SE. Proliferation data are from a single experiment that is representative of at least three independent experiments.

resulted in a highly significant enrichment of genes encoding proteins involved in cytoskeletal remodeling ($P = 2 \times 10^{-6}$), adhesion ($P = 4 \times 10^{-6}$), metaphase checkpoint ($P = 7 \times 10^{-6}$), and cell cycle regulation ($P = 7 \times 10^{-5}$) pathways. Genes in these pathways and other biologically relevant genes were grouped in five categories: chemokines, complement, cytokines, growth factors, and immunosuppression (Fig. 8). These categories highlight significant differences between MØ and this new subpopulation of regulatory Foxp3⁺ MØ. Most importantly, the microarray analysis confirmed that genes related to immune suppression, such as Foxp3, Arg2, IL10, Tnfsfr18 (GITR), and others, were also up-regulated in Foxp3⁺ MØ. Indeed, GeneGo Metabolic Networks analysis identified the 1,2-didocosahexaenoyl-sn-glycerol-3-phosphate pathway, which leads to arachidonic acid (a PGE_2 precursor) biosynthesis, as the most significantly enriched metabolic pathway ($P = 2 \times 10^{-7}$). This also corroborates our previous results showing heightened PGE_2 production by these cells (Fig. 8). In addition, we examined the transcriptional similarities between Foxp3⁺ MØ and T reg cells. A transcriptional profile of double-sorted natural T reg cells was generated and 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 ($P = 8 \times 10^{-5}$) and signaling through the TGF-βR family ($P = 1 \times 10^{-4}$). 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

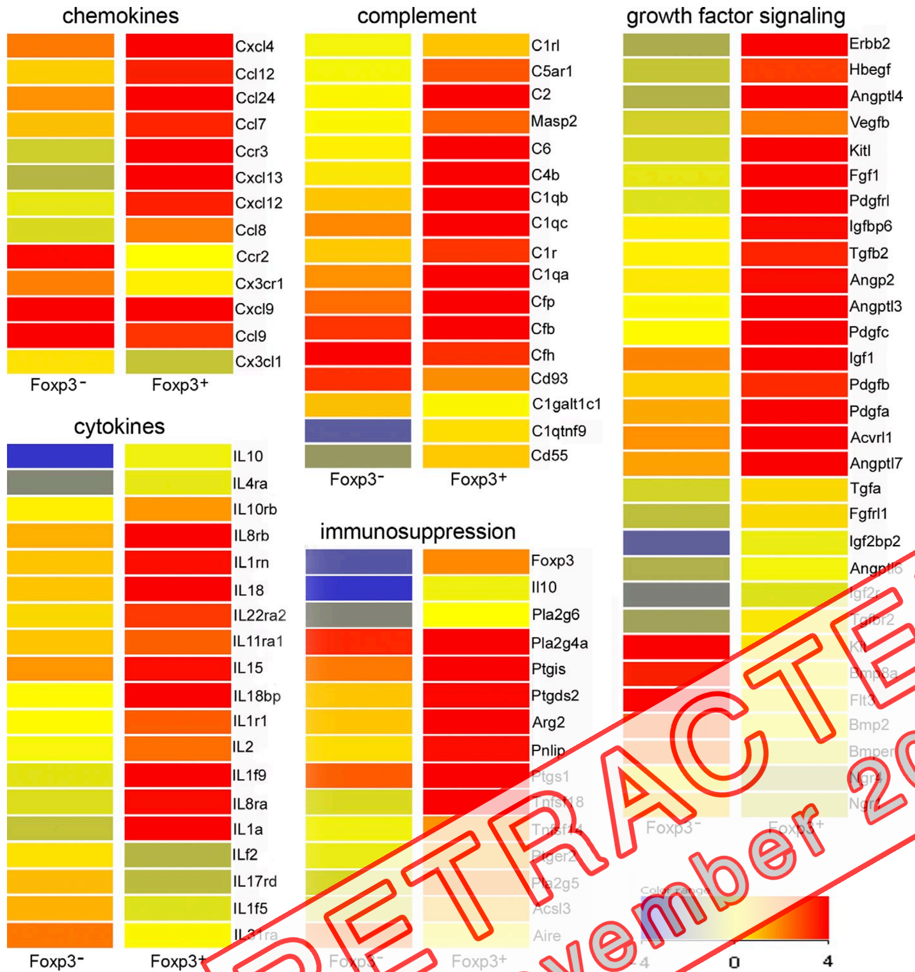

Figure 8. Global transcriptional analysis of CD11b⁺F4/80⁺Foxp3⁻ and CD11b⁺F4/80⁺Foxp3⁺ cells. Heat maps showing five groups of gene categories derived from significantly enriched pathways ($P < 0.001$), illustrating major differences in gene expression levels between double-sorted F4/80⁺Foxp3⁺ and double-sorted F4/80⁺Foxp3⁻ cells. All genes are significantly ($P = 0.05$) differentially expressed with greater than or equal to twofold change. The color bar shows ranges from −4 to +4 on a log2 scale.

tumor growth and progression (Mills, 2001). We investigated whether there were differences in the levels of Arg1 and inducible NO synthase (iNOS) between F4/80⁺Foxp3⁻ and F4/80⁺Foxp3⁺ cells. Our results indicate that higher percentages of single-sorted F4/80⁺Foxp3⁺ cells were positive for Arg1, whereas both F4/80⁺Foxp3⁻ and F4/80⁺Foxp3⁺ cells minimally expressed iNOS (Fig. 9 A).

The production of cytokines, chemokines, and growth factors secreted by F4/80⁺Foxp3⁻ and F4/80⁺Foxp3⁺ cells was also studied (Fig. 9 B). Our results showed that differences of at least twofold or more existed between F4/80⁺Foxp3⁻ and F4/80⁺Foxp3⁺ cells. F4/80⁺Foxp3⁻ cells secreted more IL12(p40), IL12(p70), IL13, IL15, TNF, and TGF-β1, whereas F4/80⁺Foxp3⁺ cells secreted more IL-1α, CXCL2, CCL4, CXCL1, VEGF, and platelet-derived growth factor (PDGF) BB. Interestingly, the basal levels of TGF-β1 secreted by F4/80⁺Foxp3⁻ cells were ∼20-fold higher than those produced by F4/80⁺Foxp3⁺ cells. As shown in Fig. 7 A, TGF-β induces the conversion of F4/80⁺Foxp3⁻ cells into Foxp3⁺ cells. Because F4/80⁺Foxp3⁻ cells produce high levels of TGF-β, it can be hypothesized that the production of TGF-β by F4/80⁺Foxp3⁻ cells could induce their conversion into Foxp3⁺ MØ. Our data indicate that the conversion of Foxp3⁺ MØ is dose dependent and the conversion of large numbers of F4/80⁺Foxp3⁺ cells requires high concentrations of TGF-β (Fig. S4). 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. 10 A). 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. 10 B). 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. 10 B). 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).

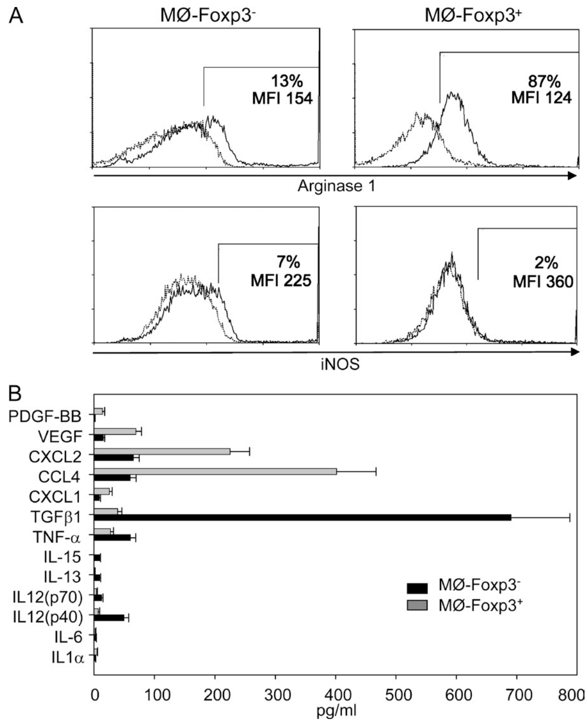

Figure 9. Analysis of cytokine profiles of CD11b$^+$F4/80$^+$Foxp3$^+$ and CD11b$^+$F4/80$^+$Foxp3$^-$ cells. (A) The level of Arginase 1 and iNOS expression (thin line) in single-sorted F4/80$^+$Foxp3$^-$ and F4/80$^+$Foxp3$^+$ cells was evaluated by flow cytometry. Control antibody, dotted line. Data represent one experiment of at least three separate experiments. (B) Single-sorted F4/80$^+$Foxp3$^+$ and F4/80$^+$Foxp3$^-$ cells were cultured for 3 d in complete medium supernatants collected and the levels of cytokines, chemokines, and growth factors were analyzed by multiplex assay. Each value represents the mean of triplicate wells ± SE.

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 MØ 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 MØ 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

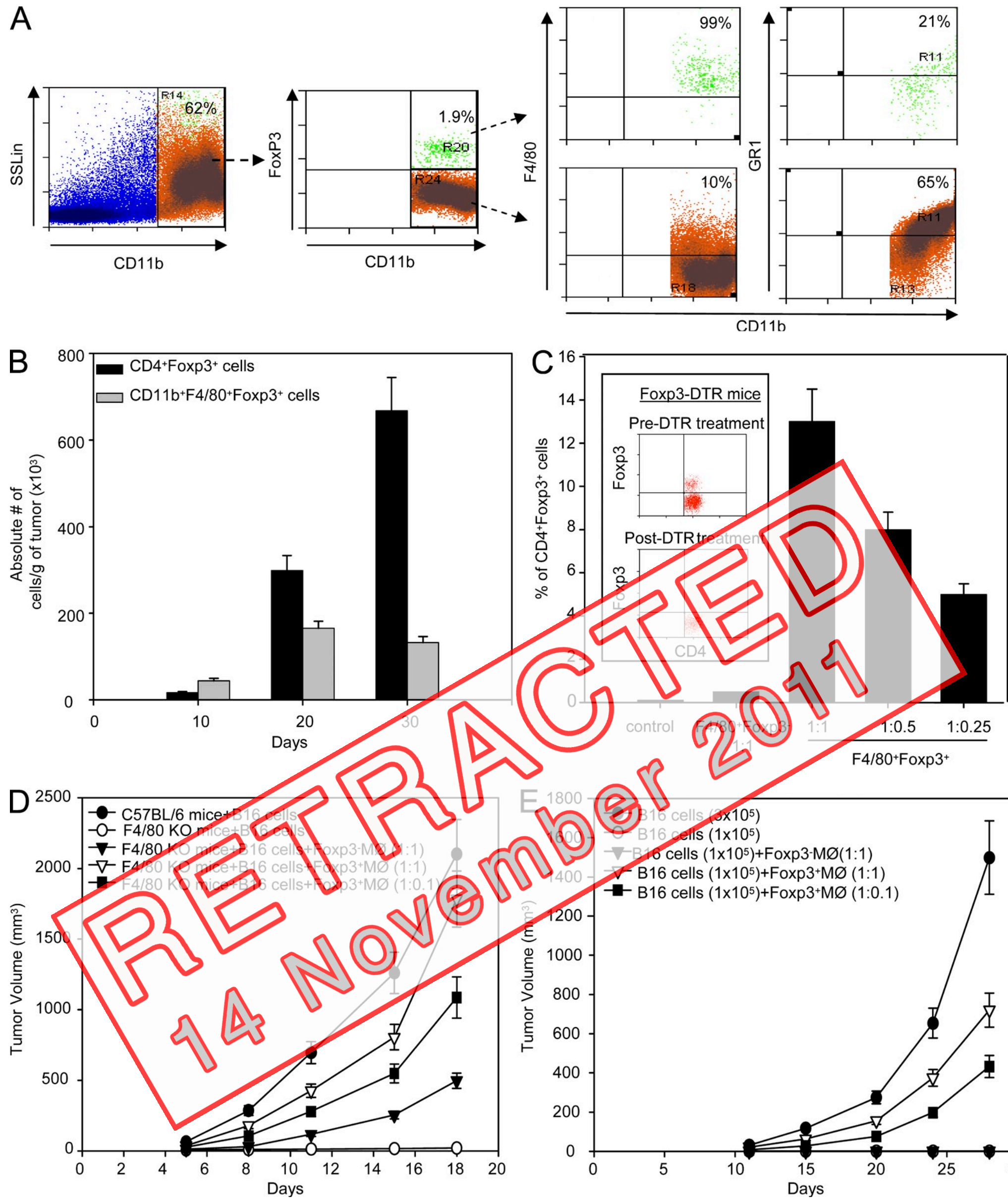

Figure 10. CD11b$^+$F4/80$^+$Foxp3$^+$ cells promote tumor growth. (A) A 30-d-old B16 tumor was stained with anti–CD11b-APC, anti–Foxp3-FITC, and anti–F4/80-PE or anti–GR1-PE mAb. CD11b$^+$ cells were gated and percentages of CD11b$^+$Foxp3$^+$ cells were determined. Percentages of double-positive CD11b/F4/80 or CD11b/GR-1 cell were determined from the gated CD11b$^+$/Foxp3$^+$ 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 1×10^6 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 1×10^6 B16 cells. Single-sorted CD11b$^+$F4/80$^+$Foxp3$^+$ cells from Foxp3-GFP mice (10^6 or 10^5) were mixed with B16 cells (10^6) 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 10^6 B16 cells and 10^6 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) 10^5 single-sorted F4/80$^+$Foxp3$^\pm$ or 10^4 single-sorted F4/80$^+$Foxp3$^+$ cells from Foxp3-GFP mice, mixed with 10^5 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 3×10^5 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$^+$ MØ is distributed in all major lymphoid organs. The presence of Foxp3$^+$ MØ 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 MØ. 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 MØ 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$^-$ MØ. 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 been also 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 MØ; 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. PGE_2 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 PGE_2. These results show that PGE_2 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$^+$ MØ compared with Foxp3$^-$ MØ (unpublished data). Therefore, it could be hypothesized that Foxp3$^+$ MØ 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 MØ 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$^-$ MØ are distinct subpopulations of MØ 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$^-$ MØ. The high expression in Foxp3$^+$ MØ of genes, such as Foxp3, IL-4R, Arg2, various phospholipases (Pla2g family), and others, correlates 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$^+$ MØ. Genes involved in prostaglandin production (e.g., PTGS1 [cyclooxigenase-1], Pla2g4c, Plag5, and Pla2g6) are also up-regulated in Foxp3$^+$ MØ, which correlates with the mechanism of inhibition used by these cells. Foxp3$^+$ MØs 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$^+$ MØs 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 MØ 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$^+$ MØ. 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$^+$ MØ than in Foxp3$^-$ MØ. 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$^+$ MØ than in Foxp3$^-$ MØ but similar to levels in T reg cells. These are examples indicating some transcriptional concordance between T reg cells and Foxp3$^+$ MØ, 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$^+$ MØ.

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-1α, CXCL4, CCL7, CCL9, CXCL12, CXCL13, PDGF, and VEGF. Arginase promotes tumor growth and progression (Bronte and Zanovello, 2005). Secretion of IL-1α 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$^+$ MØ 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$^+$ MØ 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-MØ and F4/80$^+$FoxP3$^-$ cells to M1-MØ 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-MØ, whereas F4/80$^+$FoxP3$^-$ cells resemble M1-MØ. It is important to emphasize that the distinction of M1 and M2 MØ 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$^+$ MØ that expresses Foxp3. The expression of Foxp3 confers to these MØs 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 (Schepens 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 mAb. 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. 10^5 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 ^{3}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 ^{3}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 $CD11b^+F480^+FOXP3^+$, $CD11b^+F480^+FOXP3^-$ (bone marrow), and T reg (spleen) 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′-TTGGCCAGCGCCATCTT-3′; foxp3 reverse, 5′-TGCCTCCTCCAGAGAGAAGTG-3′) and GAPDH primers (GAPDH forward, 5′-ACCCAGAAGACTGTGGATGG-3′; GAPDH reverse, 5′-CACATTGGGGGTAGGAACAC-3′) were used to determine FOXP3 mRNA levels in each population.

PGE_2 production. Analysis of PGE_2 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 PGE_2.

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 (eBioscience) and analyzed by flow cytometry. To evaluate, arginase and iNOS antibodies were purchased from BD.

Conversion analysis. 1×10^5 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 luminescent 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. 1.65 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. GSE23793 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23793)

Tumor growth analysis. C57BL/6 mice were implanted with 10^6 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. 10^6 or 10^5 single-sorted $CD11b^+F4/80^+Foxp3^{+/-}$ cells were mixed with 10^6 B16 cells and implanted s.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 $Foxp3^+$ from spleen cells and their phenotypic characterization. Fig. S4 shows titration of TGF-β for the induction of $Foxp3^+$ MØ. 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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