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Tim-3/Galectin-9 Pathway: Regulation of Th1 Immunity through Promotion of CD11b+Ly-6G+ Myeloid Cells

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IFN-γ plays a central role in antitumor immunity. T cell Ig and mucin domain (Tim-3) is expressed on IFN-γ-producing Th1 cells; on interaction with its ligand, galectin-9, Th1 immunity is terminated. In this study, we show that transgenic overexpression of Tim-3 on T cells results in an increase in CD11b+Ly-6G+ cells and inhibition of immune responses. Molecular characterization of CD11b+Ly-6G+ cells reveals a phenotype consistent with granulocytic myeloid-derived suppressor cells. Accordingly, we find that modulation of the Tim-3/galectin-9 (Gal-9) pathway impacts on tumor growth. Similarly, overexpression of Tim-3 ligand, Gal-9, results in an increase in CD11b+Ly-6G+ cells and inhibition of immune responses. Loss of Tim-3 restores normal levels of CD11b+Ly-6G+ cells and normal immune responses in Gal-9 transgenic mice. Our data uncover a novel mechanism by which the Tim-3/Gal-9 pathway regulates immune responses and identifies this pathway as a therapeutic target in diseases where myeloid-derived suppressor cells are disadvantageous. The Journal of Immunology, 2010, 185: 1383–1392.

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fter encountering specific Ag, naive T cells activate, expand, and differentiate into various effector T cell subsets such as, Th1, Th2, and Th17, which are characterized by distinct patterns of cytokine secretion. These T cell subsets have specific effector functions and recruit different cell types as a result of the cytokines they produce. Th1 cells produce IFN-γ, Th2 cells produce IL-4, IL-5, IL-13, and Th17 cells produce IL-17, IL-21, and IL-22. Th1 cells are critical in protection against intracellular pathogens and have long been associated with the pathogenesis of many organ-specific autoimmune diseases as IFN-γ was found to be present in the target organ during the peak of disease and Th1 cells were shown to adoptively transfer disease (reviewed in Ref. 1). Moreover, IFN-γ plays a central role in the immune response to tumors (reviewed in Ref. 2). Although it is clear that IFN-γ can be immune stimulatory in that it upregulates the expression of MHC and activates macrophages and neutrophils, IFN-γ has also been suggested to have immunosuppressive properties. Indeed, IFN-γ–producing Th1 cells; IFN-γ–secreting Th1 cells (15). Similarly, Tim-3 Ig-treated mice and Tim-3−/− mice are also refractory to allograft tolerance induced by donor-specific transfusion and anti-CD40L Ab or CTLA-Ig treatment (17). Based on these data, it is clear that Tim-3/Tim3-ligand interactions are important in the suppression of immune responses induced by different tolerizing regimens; however, the mechanism is not clear. To study more precisely the mechanism by which Tim-3 on T cells induces tolerance in the immune system, we generated mouse through Promotion of CD11b+Ly-6G+ Myeloid Cells

Abbreviations used in this paper: CBA, cytometric bead array; EAE, experimental autoimmune encephalomyelitis; Gal-9, galectin-9; MDSC, myeloid-derived suppres-
sor cell; SP, single positive; Tg, transgenic; Tim-3, T cell Ig and mucin domain; TNP, trinitrophenyl; WT, wild-type.

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restores normal immune responses. Thus, the Tim-3/Gal-9 pathway regulates Th1 immune responses through at least two mechanisms; directly, by triggering cell death in Th1 cells and, indirectly, through the expansion of suppressive CD11b<sup>Ly-6G</sup><sup>+</sup> cells.

### Materials and Methods

#### Mice

For the generation of Tim-3 Tg mice, the full-length cDNA of Tim-3 (BALB/c strain) was cloned into the human CD2 expression cassette (19) and the construct microinjected directly into C57BL/6 oocytes. Mice expressing Gal-9 under the control of the β-actin promoter (18) and Tim-3<sup>b</sup>/<sup>b</sup> (16, 17) mice are on the BALB/c background and were described previously. All animals were housed according to the guidelines established by the Harvard Committee on Animals.

**Flow cytometry**

Single-cell suspensions from thymus, lymph node, or spleen were prepared and stained with the indicated Abs. Spleens were subjected to digestion with collagenase D (Roche, Basel, Switzerland). Anti-CD4, CD8, Tim-3 (8B, 2C12), CD11b, CD44, CD45, CD62L, F4/80, Ly-6G, and Ly-6C Abs were purchased from eBiosciences (San Diego, CA) and BD Biosciences (San Jose, CA). Unless otherwise noted, Ab clone 1A8 was used for Ly-6G staining. All flow cytometry data were collected on a BD FACS Calibur or LSRII (BD Biosciences) and analyzed with FlowJo Software (Tree Star, Ashland, OR).

**In vitro T cell proliferation and measurement of cytokines**

Lymphocytes were cultured in triplicate with soluble anti-CD3 in the presence of irradiated APCs. For some experiments, CD11b<sup>+</sup>CD11c<sup>+</sup> cells were sorted by flow cytometry and used as APCs. At 48 h, supernatants were collected for the measurement of cytokines and plates pulsed with [H]thymidine and harvested 16 h later. Cytokines were measured from culture supernatants by ELISA or cytometric bead array (CBA) (BD Biosciences).

**Tumor experiments**

EL4 thymoma cells (from C57BL/6) and 4T1 mammary adenocarcinoma (from BALB/c) were cultured at 37°C under 10% CO<sub>2</sub> in RPMI 1640 medium supplemented with 10% FCS, penicillin, streptomycin, and 1 mM pyruvate. WT or Tim-3 Tg C57BL/6 mice were injected with 5×10<sup>5</sup>–1×10<sup>6</sup> EL4 cells within the right flank. A total of 1×10<sup>7</sup> 4T1 cells were injected into mammary tissue of Tim-3<sup>b</sup>/<sup>b</sup> or WT BALB/c mice. Tumor surface was monitored in two dimensions three times per week using calipers. In some experiments, mice were treated with 100 µg of either anti-Tim-3 Ab (clone 5D12), anti-Ly-6G Ab (clone 1A8), or isotype controls i.p.

**Induction and assessment of experimental autoimmune encephalomyelitis**

The 6-wk-old WT or Tim-3 Tg littermates were injected s.c. with 100 µg MOG 35–55 peptide (MEGVRWYSPSRSVVVLYRNGK) emulsified in CFA (Difco Laboratories, Detroit, MI) supplemented with 4 µg ml<sup>−1</sup> Mycobacterium tuberculosis and injected twice i.v. with 100 ng pertussis toxin (List Biological Laboratories, Campbell, CA). Clinical assessment of experimental autoimmune encephalomyelitis (EAE) was as follows: 0, no disease; 1, decreased tail tone; 2, hindlimb paresis; 3, complete hindlimb paralysis; 4, forelimb and hindlimb paralysis; and 5, moribund state.

**Analysis of CD11b<sup>+</sup> cells in the CNS**

EAE was induced in Tim-3 Tg mice and WT littermates by immunization with 100 µg MOG 35–55 emulsified in CFA (Difco Laboratories) supplemented with 4 µg ml<sup>−1</sup> M. tuberculosis and injected twice i.v. with 100 ng pertussis toxin (List Biological Laboratories). At different stages of disease, mice were sacrificed and CNS mononuclear cells obtained by Percoll gradient centrifugation of collagenase-digested CNS tissue (brain and spinal cord). Cells were then stained with Abs to CD11b, CD45, Ly-6G, and F4/80 and analyzed on a BD FACS Calibur.

**Immunization and assessment of recall responses to trinitrophenyl-ova**

Mice were immunized s.c. with 100 µg trinitrophenyl (TNP)-OVA in CFA. On day 10, draining lymph nodes were harvested and restimulated with TNP-OVA. Proliferation and cytokine production were measured as described previously.

### Results

#### Depressed T cell responses in Tim-3 Tg mice

We generated a Tim-3 Tg mouse by expressing the full-length Tim-3 cDNA (BALB/c isoform) under the control of the human CD2 promoter (19) on the C57BL/6 genetic background. In these mice, we are able to track Tim-3 transgene positive cells with an Ab specific for the BALB/c isoform of Tim-3 (clone 8B, 2C12), Tim-3 Tg mice are viable, fertile, and do not exhibit any gross alterations in the size of lymphoid organs. In the thymus, the Tim-3 transgene is expressed at the double negative stage and maintained through the double positive and single positive (SP) stages of thymocyte development. Interestingly, the Tim-3 transgene is expressed in only 30–40% of CD4<sup>+</sup>SP and CD8<sup>+</sup>SP thymocytes (Fig. 1A). Analysis of thymic development showed a small decrease in the frequency of Tim-3<sup>+</sup> double negative thymocytes, but no statistically significant difference in the frequency of Tim-3<sup>+</sup> double positive, CD4SP, and CD8SP thymocytes when compared with Tim-3<sup>+</sup> thymocytes (data not shown).

In the periphery, we observed no alterations in the frequency or numbers of T or B cells in Tim-3 Tg mice compared with littermate controls (data not shown). As observed in the thymus, only 30–40% of peripheral CD4<sup>+</sup> T and CD8<sup>+</sup> T cells express the Tim-3 transgene (Fig. 1A). We also examined the CD4<sup>+</sup> regulatory T cell compartment in Tim-3 Tg mice and found that neither the frequency nor the number of regulatory T cells is altered in Tim-3 Tg mice relative to littermate controls. Moreover, the Tim-3 transgene is not expressed on the regulatory T cells in Tim-3 Tg mice (Supplemental Fig. 1). We next characterized the effector/memory phenotype of peripheral CD4<sup>+</sup> T cells and found no major differences in the expression of both CD62L and CD44 in the whole CD4<sup>+</sup> T cell compartment of WT and Tim-3 Tg mice (Fig. 1B). However, when CD4<sup>+</sup> T cells from Tim-3 Tg mice were segregated into Tim-3<sup>+</sup> and Tim-3<sup>−</sup> populations, the Tim-3<sup>+</sup> T cell population contained significantly fewer effector/memory (CD4<sup>+</sup>high and CD62L<sup>low</sup>) T cells compared with the Tim-3<sup>−</sup> population (Fig. 1B), suggesting that Tim-3 expression on T cells controls effector/memory cell generation. The same trend was observed with CD4<sup>+</sup> but not CD62L<sup>+</sup> expression on CD8<sup>+</sup> T cells (data not shown).

Importantly, the CD4<sup>+</sup>Tim-3<sup>+</sup> T cell compartment was not affected in that the ratio of effector/memory (CD4<sup>+</sup>high or CD62L<sup>low</sup>) to naive (CD4<sup>+</sup>low or CD62L<sup>high</sup>) cells in these cells was similar to that of CD4<sup>+</sup> T cells from WT littermate controls (Fig. 1B).

To determine the effect of Tim-3 overexpression on a limited number of T cells on the total T cell response, we stimulated total splenocytes from Tim-3 Tg mice with anti-CD3. Although Tim-3<sup>+</sup> T cells were expressed on a limited number of T cells, T cell proliferation was decreased and IFN-γ production was reduced to background levels when compared with cultures from WT littermates (Fig. 1C). These data suggested the presence of a dominant factor(s) in Tim-3 Tg mice that suppresses T cell responses.
FIGURE 1. Lymphocyte and myeloid populations in Tim-3 Tg mice. A, Tim-3 expression in Tim-3 Tg mice (open histogram) relative to WT littermate controls (shaded histogram) in CD4SP and CD8SP thymocytes and peripheral CD4+ and CD8+ T cells. B, CD62L expression and CD62L hi/lo ratio on CD4+ spleen cells from naive WT (n = 7) and Tim-3 Tg (n = 6) mice (p = 0.02, unpaired t test). CD44 expression and CD44 hi/lo ratio on CD4+ spleen cells from naive WT (n = 8) and Tim-3 Tg (n = 7) mice (p = 0.0008, unpaired t test). C, Splenocytes (5 × 10^5/well) from naive mice were stimulated with soluble anti-CD3. Error bars indicate SEM. IFN-γ production was measured by CBA. Similar results were obtained in three independent assays. D, Frequency of CD11b+ cells in naive mice, (p = 0.0159, Mann-Whitney U test). F4/80 expression on CD11b+ cells and CD11b+ Gr-1 (RB6-8C5) in spleen of WT and Tim-3 Tg mice. E, WT CD4+ T cells (5 × 10^5/well) were stimulated with soluble anti-CD3 (0.5 μg/ml) and sorted CD11b+ cells (5 × 10^5/well) from either Tim-3 Tg or WT littermates. Error bars indicate SEM. IFN-γ production was measured by CBA. Similar results were obtained in an independent assay.
Alterations in the CD11b+ compartment in Tim-3 Tg mice

As APCs are an integral component of T cell proliferation, we examined the APC compartment in Tim-3 Tg mice for alterations. We found no significant difference in the frequency or number of CD11c+ dendritic cells or CD19+ B cells (data not shown). However, we did find that the CD11b+ population in Tim-3 Tg mice was double the population of WT littermates (Fig. 1D). As the CD11b+ population contains many cell types, including monocyte/macrophages and granulocytes, we further characterized the phenotype of the expanded CD11b+ cells in Tim-3 Tg mice by staining for F4/80, a marker for monocyte/macrophages and eosinophils and Gr-1, a marker for inflammatory monocytes and granulocytes. We found that the proportion of CD11b+ cells that are Gr-1+ and F4/80low is increased in Tim-3 Tg mice relative to WT littermates (Fig. 1D). Absolute numbers of CD11b+Gr-1+ are increased ∼2- to 3-fold in Tim-3 Tg mice relative to WT littermates (0.65–2.2×106 in WT littermates and 1.4–6.7×106 in Tim-3 Tg mice). To test the functional consequences of an increase in this population for T cell activation, we compared the ability of the total CD11b+ population from Tim-3 Tg mice and WT mice to stimulate T cell proliferation and found that the total CD11b+ population from Tim-3 Tg mice suppressed both proliferation and IFN-γ secretion from WT CD4+ T cells (Fig. 1E). Collectively, these data suggest that the expansion of CD11b+Gr-1+F4/80low cells is associated with dampened T cell immunity in Tim-3 Tg mice.

CD11b+Gr-1+ cells have been shown to arise in tumor-bearing mice and their presence is associated with poor clinical outcome (reviewed in Refs. 20–22). Accordingly, we examined the expansion of CD11b+Gr-1+ cells in the spleens of WT and Tim-3 Tg mice implanted with the T cell lymphoma, EL-4. We found Tim-3 Tg mice exhibited more CD11b+Gr-1+ cells in the spleen compared with WT littermates (Fig. 2A). In keeping with the higher expansion of CD11b+Gr-1+ cells in Tim-3 Tg mice, EL-4 tumors grew more rapidly in Tim-3 Tg mice (Fig. 2A). Moreover, treatment of EL-4 tumor-bearing mice with anti–Tim-3 Ab resulted in delayed tumor progression coincident with lower frequency of CD11b+Gr-1+ cells (Fig. 2B). As we have Tim-3−/− mice on the BALB/c background, we examined the growth of a BALB/c tumor, 4T1 mammary adenocarcinoma, in WT and Tim-3−/− mice and found that tumor progression was significantly delayed in Tim-3−/− mice and this was again coincident with lower frequency of CD11b+Gr-1+ cells (Fig. 2C).

**FIGURE 2.** Modulation of Tim-3/Gal-9 pathway in tumor-bearing animals. A, Frequency of CD11b+Gr-1+ cells in the spleens of naive (n = 6) and tumor-bearing WT (n = 11) and Tim-3 Tg mice (n = 10). (* and ** denote p < 0.001, ***p < 0.05, one-way ANOVA, Tukey’s multiple comparison test). Left panel, 1×10⁶ EL-4 cells were implanted in Tim-3 Tg (n = 5) and WT littermate controls (n = 5) (*p < 0.038, ***p < 0.0099, unpaired t test). Right panel, 5×10⁶ EL-4 cells were implanted in Tim-3 Tg (n = 4) and WT littermate controls (n = 4) (**p = 0.049, unpaired t test). Error bars indicate SEM. B, A total of 1×10⁶ EL-4 cells were implanted in WT B6 mice. Mice were then treated with mouse IgG1 or anti–Tim-3 (clone 5D12) (**p = 0.04, unpaired t test). Frequency of CD11b+Gr-1+ cells in the spleen of naive (n = 6) and tumor-bearing mice treated with mouse IgG1 (n = 10) and anti–Tim-3 (n = 11) (**p < 0.001, one-way ANOVA, Tukey’s multiple comparison test). C, A total of 1×10⁶ 4T1 cells were implanted in mammary tissue of Tim-3−/− and WT controls (**p < 0.027, ***p = 0.0078, ***p = 0.0003, unpaired t test). Similar results were obtained in an independent experiment. Frequency of CD11b+Gr-1+ cells in tumor-bearing WT (n = 10) and Tim-3−/− (n = 9) mice (p = 0.0023, unpaired t test). D, A total of 1×10⁶ EL-4 cells were implanted in WT B6 mice that were then treated with anti–Ly-6G Ab (clone 1A8) or RatIgG2a (*p = 0.0195, ***p = 0.0046, unpaired t test).

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Because the Gr-1 Ag consists of epitopes from both Ly-6G and Ly-6C, we further examined the expression of these two molecules with specific Abs and found that the proportion of CD11b<sup>+</sup> cells that are Ly-6G<sup>+</sup> and Ly-6C<sup>low</sup> is higher in Tim-3 Tg mice relative to WT littermates (Supplemental Fig. 2). Thus, Tim-3 Tg mice have more CD11b<sup>+</sup> cells that are Ly-6G<sup>F4/80</sup>low Ly-6C<sup>low</sup>. To further examine the nature of the CD11b<sup>+</sup> cells that are expanded in Tim-3 Tg mice, we isolated the CD11b<sup>+</sup>Ly-6G<sup>F4/80</sup>low cells and compared their morphology to that of CD11b<sup>+</sup>Ly-6G F4/80<sup>high</sup> cells. We found that CD11b<sup>+</sup>Ly-6G<sup>F4/80</sup>low cells contained cells with complex nuclear morphology, including immature cells with ring-shaped nuclei with a wide cytoplasmic center (Supplemental Fig. 3). This nuclear morphology has been previously associated with immature myeloid cells that are suppressive (10, 23, 24). In contrast, CD11b<sup>+</sup> Ly-6G<sup>F4/80</sup>high cells consisted of cells with classic monocyte and eosinophil morphology. Although the morphology of the CD11b<sup>+</sup> Ly-6G<sup>F4/80</sup>low cells was similar in WT and Tim-3 Tg mice, both the frequency of this population and the frequency of immature cells with ring-shaped nuclei within this population was higher in Tim-3 Tg mice than in WT mice. Furthermore, the gene expression profile of CD11b<sup>+</sup>Ly-6G<sup>F4/80</sup>low cells in both WT and Tim-3 Tg mice showed striking similarities to that of CD11b<sup>+</sup> cells isolated from tumor-bearing mice (9) (Supplemental Fig. 4).

We therefore examined the effect of anti–Tim-3 Ab on the expansion of CD11b<sup>+</sup>Ly-6G<sup>+</sup> cells in a WT host and found that CD11b<sup>+</sup>Ly-6G<sup>F4/80</sup>low cells expand less in mice treated with anti–Tim-3 relative to mice treated with isotype control (Supplemental Fig. 5). This is in keeping with our observation that CD11b<sup>+</sup> Gr-1<sup>+</sup> cells expand less in anti–Tim-3 treated tumor-bearing mice (Fig. 2B). We therefore tested the effect of depletion of Ly-6G<sup>+</sup> cells in EL-4 tumor-bearing mice and found that mice treated with anti–Ly-6G Ab exhibited delayed tumor growth relative to mice treated with isotype control Ab (Fig. 2C). Collectively, these data suggest that interference with Tim-3 signaling, either in Tim-3 Tg mice, mice treated with anti–Tim-3 Ab, or in Tim-3<sup>−/−</sup> mice, impacts on tumor progression by altering the expansion of CD11b<sup>+</sup>Ly-6G<sup>F4/80</sup>low cells, which then negatively impacts on T cell activation.

**Association of CD11b<sup>+</sup>Ly-6G<sup>F4/80</sup>low cells with suppressed autopathogenic T cell responses**  

Given that Tim-3 Tg mice exhibit an increase in CD11b<sup>+</sup>Ly-6G<sup>F4/80</sup>low cells and defects in polyclonal T cell responses in vivo, we next examined how the generation of autopathogenic immune responses in vivo would be affected in Tim-3 Tg mice. We therefore immunized Tim-3 Tg mice for the development of EAE, a model of CNS autoimmunity in which myelin-specific effector T cells play a central role in disease induction. We found that Tim-3 Tg mice are protected against EAE in that the incidence of clinical disease in Tim-3 Tg mice is a third of that of WT littermates (Fig. 3A, 3B, Table I). Given the expansion of CD11b<sup>+</sup>Ly-6G<sup>F4/80</sup>low cells we had observed in the periphery of Tim-3 Tg mice, we hypothesized the resistance to autoimmune disease in Tim-3 Tg mice was due to the infiltration of these cells from the periphery into the CNS, where they could suppress effector T cell-mediated inflammation and demyelination. We therefore examined the frequency of CD11b<sup>+</sup>Ly-6G<sup>F4/80</sup>low cells infiltrating the CNS in both WT and Tim-3 Tg mice with and without disease. We found that the presence of CD11b<sup>+</sup>Ly-6G<sup>F4/80</sup>low cells in the CNS infiltrate of mice immunized for EAE correlated with the absence of clinical disease and that the frequency of these cells in the CNS infiltrate drops dramatically in diseased mice (Fig. 3C, 3D). Because Tim-3 Tg mice have more CD11b<sup>+</sup>Ly-6G<sup>F4/80</sup>low cells, more Tim-3 Tg than WT mice were disease free (n = 6 versus n = 3, respectively, Fig. 3D). Collectively, these data support a model in which Tim-3 inhibits the generation of pathogenic T cells directly.

**FIGURE 3.** CNS autoimmunity and CD11b<sup>+</sup>Ly-6G<sup>F4/80</sup>low cells in Tim-3 Tg mice. A, Tim-3 Tg (n = 4) and WT littermates (n = 4) were immunized with 100 μg MOG 35–55 in CFA and administered 100 ng pertussis toxin i.v. on days 0 and 2. Immunized mice were monitored for the development of EAE. The mean clinical disease score is shown. Error bars indicate SEM. Similar results were obtained in three independent experiments. Cumulative data are presented in Table I. B, Linear regression curve for the experiment represented in A. The slopes are significantly different (p < 0.0001). The 95% CI for each curve is represented with dashed lines. C, Ly-6G (1A8) and F4/80 expression on CD11b<sup>+</sup> CD45<sup>+</sup> cells from the CNS of a Tim-3 Tg mouse free of disease (clinical score = 0; open histogram) and a WT mouse with disease (clinical score = 3; shaded histogram). D, Frequency of F4/80<sup>+</sup>Ly-6G<sup>+</sup> Ly-6G<sup>F4/80</sup>low cells and F4/80<sup>+</sup> cells in the CD11b<sup>+</sup>CD45<sup>+</sup> population from the CNS of Tim-3 Tg (n = 0) and WT mice (n = 4) with disease (clinical score = 3) and Tim-3 Tg (n = 6) and WT mice (n = 3) without disease (clinical score = 0). Error bars indicate SEM (⁎p = 0.0028, **p = 0.0062, ***p = 0.0002, unpaired t test compared with WT mice with no disease).
by promoting the termination of Th1 cells and also indirectly by preventing the expansion of CD11b+Ly-6G+F4/80low cells that suppress the generation of autopathogenic T cell responses. Given that the majority of T cells in Tim-3 Tg mice do not express the Tim-3 transgene and thus are not subject to Gal-9–mediated regulation, these data further suggest that the role of Tim-3 in suppressing autopathogenic T cell responses through the promotion of CD11b+Ly-6G+F4/80low myeloid cells is dominant.

_T cell expression of Tim-3 promotes the expansion of CD11b+ Ly-6G+F4/80low cells_

To address whether Tim-3 expression on T cells triggers the expansion of CD11b+Ly-6G+F4/80low cells in vivo, we isolated CD4+ T cells from Tim-3 Tg+ and CD4+Tim-3 Tg- mice and transferred them into Rag1−/− recipients, which lack T and B cells but have an intact myeloid compartment. After 4–6 wk, the extent of reconstitution as determined by splenic cellularity was not different between recipients of CD4+Tim-3 Tg+ and CD4+Tim-3 Tg− T cells (Fig. 4A); however, Rag1−/− mice reconstituted with CD4+ Tim-3 Tg+ T cells contained significantly higher percentages of CD11b+ cells (Fig. 4B). When we further characterized the CD11b+ cells present in the Rag1−/− reconstituted mice, we found that the mice reconstituted with Tim-3 Tg+ T cells had a significantly higher frequency of CD11b+ cells that were Ly-6G+ and F4/80low (Fig. 4C). Consistent with this observation, the absolute number of CD11b+Ly-6G+F4/80low was higher in the Rag1−/− mice reconstituted with Tim-3 Tg+ T cells compared with Tim-3 Tg− T cell reconstituted mice (Fig. 4D). These data directly showed that increased expression of Tim-3 on T cells promotes expansion of CD11b+ Ly-6G+F4/80low cells.

_T cell responses in Gal-9 Tg mice_

Because Gal-9 is a ligand for Tim-3, we next examined whether Gal-9 is similarly involved in the expansion of CD11b+Ly-6G+F4/80low cells that suppress T cell responses. If Gal-9 is involved, then overexpression of Gal-9 should phenocopy overexpression of Tim-3. We therefore examined the ability of T cells from Gal-9 Tg mice to proliferate and produce cytokines. Although proliferation in response to anti-CD3 was not different, we observed a significant decrease in IFN-γ production with a concomitant increase in IL-10 and IL-4 production in cells from Gal-9 Tg mice compared with WT littermate controls (Fig. 5A). We next addressed the in vivo relevance of these findings by immunizing Gal-9 Tg mice and WT littermates with TNP-OVA and found a dramatic decrease in the ability of cells from immunized Gal-9 Tg mice to proliferate and produce IFN-γ on in vitro reactivation (Fig. 5B). IL-4 and IL-10 were not detected because the mice were immunized with CFA. Similar to Tim-3 Tg mice, in Gal-9 Tg mice, there was a significant decrease in the CD4+CD62Llow effector/memory T cell population (Fig. 5C). Thus, Gal-9 Tg mice appear to phenocopy Tim-3 Tg mice because there is a marked decrease in the ability of Gal-9 Tg mice to prime Th1 immune responses and generate effector/memory cells.

Expression of Tim-3 ligand, Gal-9, promotes the expansion of CD11b+Ly-6G+ cells

Because our observation that defective T cell responses are associated with an expansion of CD11b+Ly-6G+F4/80low cells in Tim-3 Tg mice, we examined whether these cells are similarly expanded in Gal-9 Tg mice. Indeed, we observed a significant increase in the CD11b+ myeloid population in Gal-9 Tg mice but no significant differences in the numbers/frequency of CD11c+ dendritic cells or CD19+ B cells (Fig. 5D, data not shown). Further characterization of the CD11b+ cells from Gal-9 Tg mice revealed an increase in the proportion of CD11b+Ly-6G+F4/80low cells in Gal-9 Tg mice (Fig. 5D). Altogether, these data demonstrate a significant expansion of CD11b+Ly-6G+F4/80low cells in mice overexpressing Gal-9 and suggest an important role for Gal-9 in the promotion of these cells.

To determine whether the expansion of CD11b+Ly-6G+F4/80low cells may be responsible for the defect in priming Th1 immune responses in Gal-9 Tg mice, we compared the ability of the total CD11b+ population obtained from Gal-9 Tg mice and WT littermates to activate WT T cells. We observed a marked decrease in proliferative responses with CD11b+ cells from Gal-9 Tg mice (Fig. 5E). This was accompanied by an almost total absence of IFN-γ production with a concomitant increase in IL-4 and IL-10 production (Fig. 5E). Collectively, these data suggest that the expansion of CD11b+Ly-6G+F4/80low cells within the total CD11b+ population could be responsible for the defect in productive Th1 immune responses observed in Gal-9 Tg mice in response to immunization.

Gal-9/Tim-3 interaction in the promotion of CD11b+Ly-6G+ F4/80low cells

To understand how Tg expression of both Tim-3 and its ligand, Gal-9, could independently result in the expansion of CD11b+Ly-6G+F4/80low cells, we examined the expression of Gal-9 by CD11b+Ly-
6G+ cells and found that they naturally express more Gal-9 than CD11b+Ly-6G+2 cells and that this difference is further augmented by activation of these cells with the Th1 cytokine IFN-γ (Fig. 6).

The higher expression of Gal-9 on CD11b+Ly-6G+ cells provides a basis for the ready interaction of Tim-3 expressing T cells with Gal-9 for the expansion of these cells. To determine whether a direct interaction of Tim-3 expressing T cells with Gal-9 is responsible for the expansion of CD11b+Ly-6G+F4/80low cells that we observe, we crossed Gal-9 Tg mice with Tim-3−/− mice. Comparison of Gal-9 Tg, WT, and Gal-9 Tg × Tim-3−/− mice revealed that the increased frequency of CD11b+ cells observed in Gal-9 Tg mice was restored to WT levels in Gal-9 Tg × Tim-3−/− mice (Fig. 7 A). Further characterization of the CD11b+ cells obtained from the different mouse strains demonstrated that the proportion of CD11b+ cells that are CD11b+Ly-6G+F4/80low is also restored to WT levels in Gal-9 Tg × Tim-3−/− mice, thereby confirming that the expansion of these cells in Gal-9 Tg mice is dependent on the interaction with Tim-3 (Fig. 7 A). Moreover, the CD11b+ cells from Gal-9 Tg × Tim-3−/− mice were equally able to stimulate proliferation and IFN-γ production from WT CD4+ T cells as CD11b+ cells taken from WT mice (Fig. 7 B). Similarly, the priming of Th1 immune responses was restored to WT levels in Gal-9 Tg × Tim-3−/− mice immunized with TNP-OVA (Fig. 7 C). Lastly, the defect in the generation of CD62Llow effector/memory cells observed in Gal-9 Tg mice after immunization was also corrected in immunized Gal-9 Tg × Tim-3−/− mice such that the Gal-9 Tg × Tim-3−/− mice had close to WT levels of effector/memory T cells (Fig. 7 D).

Discussion

Our studies have uncovered an unexpected mechanism by which the Tim-3/Gal-9 pathway controls IFN-γ–producing Th1 cells. Tim-3 is expressed by terminally differentiated Th1 cells, which then upregulate the expression of Tim-3 ligand, Gal-9, through the production of IFN-γ (25, 26). Gal-9 directly interacts with Tim-3 to inhibit the Th1-cell response by triggering cell death (15). We now show that the Tim-3/Gal-9 pathway could also indirectly regulate Th1 immune responses through the expansion of CD11b+Ly-6G+F4/80low cells. Interestingly, this cell surface phenotype is consistent with that described for granulocytic or PMN-like MDSCs (10, 27). In addition, our morphological analysis of CD11b+Ly-6G+F4/80low cells further supports that these cells are granulocytic MDSC (Supplemental Fig. 3).

MDSCs are a heterogeneous population of myeloid cells generally identified as being positive for CD11b and Gr-1 that expand in large numbers in tumor-bearing mice, cancer patients, and post-infection, trauma, or autoimmunity (reviewed in Refs. 20–22). MDSCs are potent suppressors of T cell immunity and their presence is correlated with poor clinical outcome in cancer. Recently,
MDSCs have been subdivided into two classes: monocytic (CD11b+Ly-6G$^2$Ly-6Chi) and granulocytic (CD11b +Ly-6G+Ly-6Clow) (10, 27). Both of these populations are suppressive for T cells but by different mechanisms. Monocytic MDSCs suppressed by production of NO and granulocytic MDSCs suppressed by a mechanism that involves IFN-$\gamma$ and possibly the production of reactive oxygen species as well as arginine metabolism (10, 27). Indeed, IFN-$\gamma$ has long been associated with the expansion and suppressive function of MDSCs as MDSCs fail to accumulate in IFN-$\gamma$R$^2$ / R$^2$ mice and anti–IFN-$\gamma$Ab abrogates their suppressor function (6–10). In this paper, we have made a novel observation that the Tim-3/Gal-9 pathway plays a role in the expansion and/or activation of granulocytic MDSCs, which is consistent with previous studies implicating IFN-$\gamma$ in MDSC-mediated immune suppression and that Tim-3 is a cell surface receptor expressed on IFN-$\gamma$-secreting Th1 cells.

Increased arginine metabolism acting in concert with inducible NO synthase is also implicated in MDSC-mediated immunosuppression. Consistent with this, we found Arginase II is highly upregulated in CD11b+Ly-6G$^4$/80$^{low}$ cells (Supplemental Fig. 4) and that the arginase inhibitor NOR-NOHA abrogates suppression by CD11b+ cells from Gal-9 Tg mice (Supplemental Fig 6). We also found that many genes involved in inflammation are more highly expressed in CD11b+Ly-6G$^4$/80$^{low}$ cells. In particular, we observed an upregulation of many IL-1/TNF-related genes, including IL-1R type II. In this regard, IL-1R$^2$/ R$^2$ mice exhibit a defect in MDSC accumulation (31). The expression profile of the CD11b+Ly-6G$^4$/80$^{low}$ cells in our study is consistent with these observations and with the expression profile that has been reported for splenic CD11b+ cells from tumor-bearing mice (9).

Cell–cell contact has long been known to be an important component in MDSC-mediated immunosuppression (reviewed in Ref. 32). Indeed, suppression does not occur if MDSCs are separated from T cells by a semipermeable membrane, indicating the need for cell–cell contact and interaction of membrane-bound molecule(s). However, the nature of this membrane-bound molecule(s) has remained elusive. Based on our data, we propose that Tim-3 on IFN-$\gamma$–secreting T cells interacting with Gal-9 bound on MDSCs is one such cell surface receptor/ligand pathway involved in MDSC expansion/function. Our observation of the increased frequency of

**FIGURE 6.** Gal-9 expression in CD11b$^+$Ly-6G$^-$ and CD11b$^+$Ly-6G$^+$ cells. CD11b$^+$Ly-6G$^-$ and CD11b$^+$Ly-6G$^+$ cells were isolated from WT BALB/c spleen cells by cell sorting and cultured in the presence or absence of IFN-$\gamma$ (10 ng/ml, R&D Systems) for 24 h. RNA was isolated using Qiagen RNeasy and real-time PCR performed for the expression of Gal-9. Data shown are DD$^Ct$ where the expression of Gal-9 in CD11b+Ly-6G$^-$ cells cultured in media alone was used as a calibrator. Error bars indicate SEM. The mean of two independent experiments is shown.

**FIGURE 7.** Role of Gal-9–Tim-3 interaction in the promotion of CD11b$^+$Ly-6G$^+$ cells. A, Frequency of CD11b$^+$ and ratio of CD11b$^+$Ly-6G$^4$/80$^{low}$ to CD11b$^+$Ly-6G$^4$/80$^{high}$ cells in naive WT, Gal-9 Tg, and Gal-9 Tg × Tim-3$^{-/-}$ mice (*p = 0.02; **p = 0.03; ***p = 0.03, Mann-Whitney U test). B, WT CD4$^+$ (10$^5$/well) were stimulated with purified CD11b$^+$ cells from WT, Gal-9 Tg, or Gal-9 Tg × Tim-3$^{-/-}$ mice. Proliferation was measured by [$^{3}$H]thymidine incorporation and IFN-$\gamma$ production was measured by CBA. Data shown are representative of five experiments. (p = 0.006, Mann-Whitney U test). C, Lymph node cells from immunized mice were reactivated with TNP-OVA. Proliferation was measured by [$^{3}$H]thymidine incorporation and IFN-$\gamma$ production was measured by CBA (p = 0.001, Mann-Whitney U test). D, CD62L expression in CD4$^+$ lymph node cells from immunized mice. Representative flow plots are shown.
CD11b<sup>+</sup>Gr-1<sup>+</sup> cells and increased tumor growth in Tim-3<sup>Tg</sup> mice compared with WT littermates is in keeping with this. Several cytokines and growth factors produced by tumors, such as GM-CSF, have been shown to be involved in the generation of MDSCs. The Tim-3/Gal-9 pathway may act in concert with these factors to promote MDSC expansion and suppressor function.

Although our data strongly support a role for the Tim-3/Gal-9 pathway in promoting MDSCs, our data in the 4T1 tumor model clearly show that CD11b<sup>+</sup>Gr-1<sup>+</sup> cells can expand, although to a lesser degree, in Tim-3<sup>-/-</sup> mice. Thus, the Tim-3/Gal-9 pathway contributes to but is not essential for MDSC expansion. Future studies should investigate whether Tim-3<sup>+</sup> dendritic cells also promote or contribute to MDSC expansion. This will require the use of mice in which Tim-3 is deleted only in T cells or the use of the Tim-3<sup>+</supunciover Gr1<sup>-</sup> mice as recipients of Tim-3<sup>Tg</sup> vs Tim-3<sup>Tg</sup> cells. Neither of these strains is currently available.

MDSCs are present in normal individuals at a low frequency and, as stated above, accumulate in large numbers in cancer patients and in tumor-bearing mice. Expansion of MDSCs has also been observed after exposure to bacterial (24, 33), parasitic (6, 7), viral (34) Ags, and after traumatic stress (35). Therefore, MDSCs may be part of a feedback mechanism induced to prevent damage caused by prolonged or excessive inflammation mediated by Th1 cells. Thus, the Tim-3/Gal-9 pathway regulates proinflammatory Th1 cells by two mechanisms: directly by triggering cell death in Th1 T cells and indirectly through a “cross-talk” with the myeloid compartment. The value of this pathway is further underscored by the findings that Tim-3 expression is dysregulated in human autoimmune and infectious diseases. In the case of multiple sclerosis, Tim-3 expression on T cells infiltrating the CNS was reduced (36) and in HIV-infected patients, Tim-3 was found to be overexpressed on “exhausted” T cells (37). However, whether MDSCs are affected in these disease conditions was not evaluated. It is possible that part of the exhausted phenotype observed in chronic viral infections is due to an expansion of MDSCs. The identification of this mechanism not only illustrates the importance of the dynamic interplay between adaptive and innate immunity in the regulation of effector T cell responses, but also opens new avenues of investigation in MDSC biology. Because the Tim-3/Gal-9 pathway regulates IFN-γ-producing Th1 cells, which are central components in the immune response to infection, autoimmunity, and cancer, targeting this pathway may prove beneficial in multiple disease states.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

Supplementary Figure 1. Regulatory T cells in Tim-3 transgenic mice.

(A) Frequency of CD4^{+}CD25^{+} regulatory T cells in spleen of wild type (n=5) and Tim-3 transgenic (n=5) mice.  (B) Expression of Tim-3 transgene in CD4^{+}CD25^{+} regulatory T cells and CD4^{+}CD25^{-} effector T cells in wild type (shaded histogram) and Tim-3 transgenic mice (open histogram).
Supplementary Figure 2. CD11b^+Ly-6G^+Ly-6C^{low} cells are increased in Tim-3 Tg mice. Spleen cells from wild type (left panel) and Tim-3 Tg (right panel) mice were stained with antibodies against CD11b, Ly-6G (1A8), and Ly-6C (AL-21). Shown is the expression of Ly-6G and Ly-6C on CD11b^+ cells. Numbers represent frequency of the CD11b^+Ly-6G^+Ly-6C^{low} population in the CD11b^+ gate.
Supplementary Figure 3. Morphology of sorted CD11b+ populations. CD11b+Ly-6G+F4/80low and CD11b+Ly-6G'-F4/80high cells were isolated from the spleens of wild type and Tim-3 Tg mice by cell sorting (BD FACsAria) and cytocentrifuged onto slides prior to Wright-Giemsa staining (Hema 3 kit, Fisher Scientific). The Ly-6G+F4/80low cells from wild type and Tim-3 Tg mice are myeloid cells many of which have ring-shaped nuclei (black arrows). Other cells show complex nuclear morphology. The Ly-6G'-F4/80high cells are typical mononuclear cells (green arrows) and eosinophils (red arrows). Bar =20μM
Supplementary figure 4. Gene expression profiles of sorted CD11b⁺ Ly-6G⁺ myeloid cells from WT and Tim3 transgenic mice. CD11b⁺ Ly-6G⁺ F4/80low and CD11b⁺ Ly-6G⁻ F4/80high cells were sorted from wild-type (WT) or Tim3 transgenic (Tim3Tg) mice and analyzed using a whole mouse genome array (Applied Microarrays). The analysis of genes differentially expressed (FDR < 0.1, >3 fold) in CD11b⁺ Ly-6G⁺ F4/80low cells relative to CD11b⁺ Ly-6G⁻ F4/80high cells is shown. (a) Genes potentially involved in the regulation of angiogenesis and/or hematopoietic properties of myeloid cells such as growth factors, cytokines and adhesion molecules are depicted. (b) Transcripts overexpressed in CD11b⁺ Ly-6G⁺ F4/80low cells that are similar or related to transcripts previously reported to be upregulated in splenic CD11b⁺ cells from tumor-bearing mice relative to CD11b⁺ cells from naive mice (see Gallina et al., JCI 2006).
Supplementary Figure 5. Expansion of CD11b+Ly-6G+F4/80low cells in tumor-bearing mice. C57BL/6 mice were implanted with 1x10^6 EL-4 cells and treated with either mouse IgG1 (n=10) or anti-Tim-3 antibody (clone 5D12) (n=11). Frequency of CD11b+Ly-6G+F4/80low cells in the spleens of tumor-bearing mice relative to naïve mice (n=6) is shown (*p<0.001, **p<0.01, ***p<0.05, one-way ANOVA and Tukey’s multiple comparison test.)
Supplementary Figure 6. Role of Arginase in the inhibitory mechanism exerted by CD11b+ cells from galectin-9 transgenic mice. CD4+ T cells from wild type mice were cultured with CD11b+ cells from either WT or galectin-9 transgenic mice in the presence or absence of 50 μM of the arginase inhibitor NOR-NOHA. 48 hours post-activation, cells were pulsed with ³H-Thymidine and harvested 16 hours later. Data shown are representative of two independent experiments.