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Cutting Edge: Flt3 Ligand Mediates STAT3-Independent Expansion but STAT3-Dependent Activation of Myeloid-Derived Suppressor Cells

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The Flt3–Flt3 ligand (Flt3L) pathway is critically involved in the differentiation and homeostasis of myeloid cells, including dendritic cells (DC); however, its role in the expansion and function of myeloid-derived suppressor cells (MDSC) has not been determined. In this article, we describe the ability of Flt3L to expand and activate murine MDSC capable of suppressing allograft rejection upon adoptive transfer. Although Flt3L expands and augments the stimulatory capacity of myeloid DC, MDSC expanded by Flt3L have increased suppressive activity. Although STAT3 is considered the central transcription factor for MDSC expansion, inhibition and genetic ablation of STAT3 did not block, but rather augmented, Flt3L-mediated MDSC expansion. MDSC suppressive function, preserved when STAT3 inhibition was removed, was reduced by genetic STAT3 deletion. Both STAT3 inhibition and deletion reduced Flt3L-mediated DC expansion, signifying that STAT3 had reciprocal effects on suppressive MDSC and immunostimulatory DC expansion. Together, these findings enhance our understanding of the immunomodulatory properties of Flt3L.

Myeloid-derived suppressor cells (MDSC) are recently characterized innate immunoregulatory cells that expand under inflammatory conditions, such as cancer, sepsis, allograft rejection, and autoimmune (reviewed in Refs. 1 and 2). Although mouse and human MDSC exhibit considerable heterogeneity, they share the ability to induce T cell apoptosis or suppress T cell proliferation and secretion of cytokines (2, 3). In mice, MDSC are broadly identified as CD11b+Gr1+ cells, whereas cell morphology and differential surface expression of the Gr1 Ag Ly6G and Ly6C distinguish granulocytic (CD11b+Ly6G+) and monocytic (CD11b+Ly6C+) MDSC subsets (1). Expansion and activation of MDSC occur through the action of growth factors that promote myelopoiesis (4, 5) and proinflammatory cytokines (1, 5).

Flt3 (CD135; fetal liver kinase-2 [Flk2]) is a receptor tyrosine kinase expressed on hematopoietic stem cells and early precursors (6). The Flt3–Flt3 ligand (Flt3L) pathway is critically involved in dendritic cell (DC) homeostasis (7–9). Flt3L activates the transcription factor STAT3 (10), which is strongly implicated in MDSC expansion and function (1). However, the potential of Flt3L to support MDSC expansion/activation is undefined. Because of the potent ability of Flt3L to increase myeloid precursors and activate STAT3, we hypothesized that Flt3L-driven myelopoiesis would not only promote DC expansion, but also increase suppressive MDSC.

In this article, we report that Flt3L expands and activates Ly6G+ and Ly6C+ MDSC. In contrast, DC expanded by Flt3L are more stimulatory than steady-state DC. Although DC expansion by Flt3L is dependent on STAT3, surprisingly, conditional ablation of STAT3 enhances Flt3L-induced mobilization of MDSC. However, Flt3L-expanded MDSC depend on STAT3 for optimal suppressive function. Adoptive transfer of Flt3L-mobilized MDSC, but not steady-state CD11b+Gr1+ cells, prolongs fully MHC-mismatched cardiac allograft survival.

Materials and Methods

Animals and drug administration

All mice for breeding and experimentation were from The Jackson Laboratory. Eight- to twelve-week-old male BALB/c (H2Kd) or C57BL/6 (B6; H2Kb) mice were given recombinant human Flt3L (10 μg/d i.p.; Amgen) for 10 d. Mice with conditional STAT3 gene disruption were generated by interbreeding mice expressing Cre under the LysM promoter (B6.129P2-Lyz2tm1cre/J) and floxed STAT3 (B6.129S1-Stat3tm1(cre)Ifo/J) genes. The genetic background of crossed mice was verified by PCR genotyping, and littermates were used as negative controls. The STAT3 inhibitor S31-201 (5 mg/kg; Selleck Chemicals) was administered i.p., as described (11). All studies were performed under

grant. B.M.M. was supported by National Institutes of Health Institutional Training Grant T32AI74490 (to A.W.T.). Address correspondence and reprint requests to Dr. Heth R. Turnquist, Departments of Surgery and Immunology, University of Pittsburgh School of Medicine, Thomas E. Starzl Transplantation Institute, 200 Lothrop Street, Biomedical Science Tower, E1554, Pittsburgh, PA 15213. E-mail address: turnquisthr@upmc.edu

The online version of this article contains supplemental material. Abbreviations used in this article: DC, dendritic cell; Flt3L, Flt3 ligand; HO-1, heme oxygenase-1; MDSC, myeloid-derived suppressor cell; Treg, regulatory T cell.

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Flow cytometry
Cell surface and intracellular marker expression was analyzed as described (12, 13).

MLR and suppression assay
MDSC were isolated from splenocytes by positive selection with FITC anti-Ly6C, PE anti-Ly6G, or PE anti-Gr1 using anti-FITC or anti-PE MicroBeads (Miltenyi Biotec), as described (12, 14). DC were isolated by CD11c immunomagnetic bead selection and gamma irradiated (20 Gy). T cell proliferation was assessed at 72 h by [3H]TdR incorporation or CFSE dilution (Invitrogen).

Vascularized heart transplantation
Heterotopic intra-abdominal mouse heart transplantation was performed, and graft survival was monitored as described (14).

Statistics
Data are presented as mean ± 1 SD. Significant differences between means and survival curves were determined using a two-tailed, Student t test and log-rank test, respectively.

Results and Discussion
We first examined myeloid populations expanded by Flt3L. Total splenocyte number was increased (Fig. 1A) and, in agreement with previous studies (7, 9), Flt3L increased the frequency and absolute number of conventional myeloid DC (Fig. 1B–D). Splenic CD11b+Gr1+ cells also were increased in frequency and absolute number by Flt3L; however, macrophage (CD11bF4/80hi) frequency was unchanged (Fig. 1B, 1C). Of the lymphoid populations examined, Flt3L only increased the frequency of Foxp3+ regulatory T cells (Treg); however, the absolute number of all T cell populations was augmented (Supplemental Fig. 1). This increase in naturally occurring Treg is thought to be due to DC-mediated Treg expansion (15). Proinflammatory cytokines are not expected to play a significant role in modulating the incidence or frequency of cell populations, because IL-1β, IL-4, IL-6, IL-10, IL-12p40, TNF-α, and IFN-γ were not detected in the serum of PBS- or Flt3L-treated mice (data not shown). Interestingly, circulating TGF-β1 levels were reduced by 3-fold in Flt3L-treated mice (1229 ± 265.8 pg/ml in naive mice versus 441.1 ± 26.15 pg/ml in Flt3L-treated mice, p < 0.05; data not shown).

We next sought to further characterize surface Ag expression on CD11b+Ly6C+ cells expanded by Flt3L. Flt3L induced expansion of both CD11b+Ly6Cint/hi and CD11b+Ly6G+ cells (Fig. 1E–G). CD11b+Ly6Chi cells expressed an intermediate level of F4/80 and were CD115 (M-CSF receptor)+, consistent with surface Ag expression described for MDSC (Fig. 1E) (1). CD11b+Ly6Cint and CD11b+Ly6G+ cells were
F4/80− and expressed only low levels of CD115 (Fig. 1E, 1F). Solheim et al. (16) described an increase in splenic CD11b+Gr1+ cells following adenoviral delivery of Flt3L to tumor-bearing mice; however, the suppressive function of these cells was not assessed. We now show that both Ly6C+ monocytic and Ly6G+ granulocytic MDSC from Flt3L-treated mice are suppressive in MLR (Fig. 1H). Moreover, both Flt3L-expanded Ly6C+ and Ly6G+ MDSC were significantly more potent suppressors than their counterparts from steady-state control mice (Fig. 1H). In contrast, CD11c+ DC isolated from Flt3L-treated mice demonstrated increased allogeneic T cell stimulatory capacity (Fig. 1I). Thus, these data reveal that Flt3L has reciprocal capacities to expand functionally distinct populations of stimulatory DC and suppressive MDSC.

STAT3 is considered the key regulator of MDSC expansion and suppressive function, and Flt3L is a potent activator of STAT3 (10). Therefore, we next ascertained whether STAT3 is required for Flt3L-mediated MDSC expansion. Genetic ablation of STAT3 prior to Flt3L administration reduced the frequency of myeloid DC (Fig. 2A, 2B), consistent with earlier reports (10) using conditional STAT3-knockout mice. In contrast, expansion of CD11b+Gr1+ cells by Flt3L was augmented by STAT3 deletion (Fig. 2A–C). Chemical inhibition of STAT3 in vivo during Flt3L administration generated similar results (Supplemental Fig. 2). Flt3L causes an accumulation of common myeloid progenitors in conditional STAT3-knockout mice (10), which may serve as an important source of immunosuppressive MDSC. Consistent with the importance of STAT3 in GM-CSF–mediated activation (17), STAT3 deletion reduced Flt3L-expanded MDSC suppressive function (Fig. 2D). However, this did not occur when MDSC were isolated from mice treated with a STAT3 inhibitor and Flt3L (Supplemental Fig. 2F), potentially as a result of reversibility of STAT3 inhibition during ex vivo suppression. MDSC suppress T cell proliferation through several immunosuppressive enzymes, including arginase-1, inducible NO synthase, heme oxygenase-1 (HO-1), and IDO (1, 18, 19). Both steady-state control and Flt3L-mobilized Gr1+ cells independently required HO-1 and IDO for suppression of T cell proliferation (Fig. 2E) and predominantly suppressed CD4+ T cells without affecting Treg frequency (Supplemental Fig. 2G–J).

Adoptively transferred bone marrow–derived MDSC inhibit graft-versus-host disease (20), and allogeneic skin transplant–activated MDSC transferred to skin graft recipients prolong survival (21). Furthermore, MDSC are required for the induction of organ transplant tolerance by costimulation blockade (22). In the current study, Gr1+ cells isolated from splenocytes of Flt3L-treated mice, but not control mice, sig-

**FIGURE 2.** Flt3L-mobilized MDSC are expanded in a STAT3-independent manner and prolong cardiac allograft survival. (A) STAT3 conditional KO (cKO) mice were generated using LysM-Cre and floxed STAT3 mice and administered Flt3L. (B) Splenic DC and MDSC were identified by flow cytometry and quantified. (C) STAT3 deletion in isolated splenic Flt3L-expanded Gr-1+ cells was verified by Western blot. (D) Suppressive capacity of Flt3L-expanded MDSC from STAT3−/− and STAT3 cKO mice were compared in suppression assays when splenic B6 Gr1+ cells (1 × 105 or 0.25 × 105) were used as suppressors of B6 CD3+ T cells (1 × 105) stimulated with BALB/c Flt3L-mobilized CD11c+ DC (1.25 × 104). *p < 0.05, versus Flt3L. (E) Splenic BALB/c Gr1+ cells (1 × 106) were used as suppressors of BALB/c CD3+ T cells (1 × 105) stimulated with B6 Flt3L-mobilized CD11c+ DC (1.25 × 104). Inhibitors of NO synthase (N’methyl-L-arginine [L-NMMA]; 0.5 mM), arginase-1 (N-Hydroxy-nor-L-arginine [norNOHA]; 0.5 mM), HO-1 (tin protoporphyrin [SnPP]; 0.15 mM), or IDO (1-methyl-D-tryptophan [1-Me-D-trp]; 0.2 mM) was added at the start of coculture, where indicated. *p < 0.05, versus PBS or Flt3L Gr1+ cells in the absence of inhibitor, unless otherwise indicated. (F) A total of 5 × 10⁶ BALB/c Gr1+ cells was administered i.v. to BALB/c recipients 1 d before B6 heart transplant, and allograft survival was monitored. Data are representative of two independent experiments, with six or seven total mice/group. *p < 0.05.
nificantly prolonged fully MHC-mismatched cardiac allograft survival in the absence of additional immunosuppression (Fig. 2F), thus demonstrating their in vivo suppressive function. Flt3L was reported to have both pro- and anti-inflammatory effects in disease models (23–25). Thus, the varying impact of Flt3L on immune responses in vivo remains poorly understood, and the role of MDSC in these models has not been explored. Our data show that Flt3L mediates STAT3-independent expansion of suppressive MDSC but STAT3-dependent expansion of stimulatory CD11c+ DC. These data also add further support for the importance of the STAT3 pathway in the suppressive activity of cytokine-expanded MDSC.

These findings have significant clinical relevance for the use of Flt3L as an immune-modulating agent. Combination of Flt3L administration with STAT3 inhibition could promote effective immune regulation, given the expectation that STAT3 inhibition will counter Flt3L-driven DC generation but allow MDSC expansion. Additionally, our data suggest that, upon clearance of STAT3 inhibition, augmented MDSC will be functionally suppressive. Conversely, delivery of Flt3L with inhibitors of IDO or HO-1 would be expected to augment previously demonstrated immune adjuvant properties of Flt3L.

Disclosures

The authors have no financial conflicts of interest.

References

SUPPLEMENTAL FIGURE 1. Flt3L reduces the frequency of splenic B cells and pan CD4⁺ T cells, while Foxp3⁺ Treg frequency is increased. A, CD45⁺-gated BALB/c splenocytes were analyzed for the frequency of lymphoid cell subsets, including B cells (B220⁺), CD4⁺ T cells (CD3⁺CD4⁺), CD8⁺ T cells (CD3⁺CD8⁺), and Treg (CD3⁺CD4⁺Foxp3⁺). B, The frequency of lymphoid subsets was determined across multiple experiments and (C) absolute numbers quantified. Data are representative of n≥6 mice. * p<0.05 by two-tailed Student’s ‘t’ test.
SUPPLEMENTAL FIGURE 2. STAT3 inhibition during Flt3L administration reduces DC expansion, but augments expansion of Gr1+ cells that potently suppress CD4+ T cells without affecting Treg frequency. A, STAT3 inhibitor (STAT3i) was co-administered with Flt3L, and DC and MDSC frequency was determined and (B) quantified. C, Total viable splenocytes, (D) DC, and (E) Gr1+ cells were isolated and enumerated. (F) Isolated BALB/c splenic Gr1+ cells were used to suppress BALB/c CD3+ T cells (1x10^5) stimulated with B6 Flt3L-mobilized CD11c+ DC (1.25x10^4). A-F, * and # p<0.05 compared to PBS and Flt3L groups, respectively. G, MLR co-cultures were performed as described in Fig. 2E with CFSE-labeled CD3+ T cell responders. Activated CD4+ and CD8+ T cell proliferation was determined by CFSE dilution on d3 and (G-J) quantified. I-J, The frequency of CD4+Foxp3+ T cells was also determined on d5 of co-culture. * p<0.05 from n= 2-3 independent experiments with n≥4 mice per group.