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Flt3 ligand mediates STAT3-independent expansion, but STAT3-dependent activation of myeloid-derived suppressor cells

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Abstract

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. Herein, we describe the ability of Flt3L to expand and activate murine MDSC capable of suppressing allograft rejection upon adoptive transfer. While 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 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 understanding of the immunomodulatory properties of Flt3L.

Introduction

Myeloid-derived suppressor cells (MDSC) are recently-characterized innate immunoregulatory cells that expand under inflammatory conditions, such as cancer, sepsis, allograft rejection, and autoimmunity [reviewed (1, 2)]. Although mouse and human MDSC exhibit considerable heterogeneity, they share the ability to induce apoptosis or suppress T cell proliferation and secretion of cytokines (2, 3). In mice, MDSC are broadly identified as $CD11b^{+}Gr1^{+}$ cells, while cell morphology and differential surface expression of the Gr1 Ag

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Ly6G and Ly6C distinguish granulocytic ($CD11b^+Ly6G^+$) and monocytic ($CD11b^+Ly6C^+$) subsets (1). Expansion and activation of MDSC occurs through the action of growth factors that promote myelopoiesis (4, 5) and pro-inflammatory cytokines (1, 5).

Fms-like tyrosine kinase 3 [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), that is strongly implicated in MDSC expansion and function (1). However, the potential of Flt3L to support MDSC expansion/activation is undefined. Due to the potent ability of Flt3L to increase myeloid precursors and activate STAT3, we hypothesized that Flt3L-driven myelopoiesis would not only promote DC, but also suppressive MDSC.

Herein, 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 depended 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. 8–12 week old male BALB/c ($H2K^d$) or C57BL/6 (B6; $H2K^b$) mice were given r 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-Lyz2$^{tm1(cre)Ifo}$/J) and floxed STAT3 (B6.129S1-Stat3^{tm1Xyfu}/J) genes. The genetic background of crossed mice was verified by PCR genotyping and littermates 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 an institutional animal care and use committee-approved protocol.

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 γ-irradiated (20 Gy). T cell proliferation was assessed at 72 h by [^{3}H] TdR incorporation or CFSE dilution (Invitrogen).

Vascularized heart transplantation

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

Statistics

Data are presented as means ± 1 standard deviation. Significant differences between means and survival curves were determined using a two-tailed, Student's 't' test and log-rank test, respectively.

Results and Discussion

We first examined myeloid populations expanded by Flt3L. Total splenocyte number was increased (Fig. 1*A*) and, in agreement with previous studies (7, 9), Flt3L increased the frequency and absolute number of conventional myeloid DC (Fig. 1*B–D*). Splenic $CD11b^+Gr1^+$ cells were also increased in frequency and absolute number by Flt3L; however, macrophage ($CD11b^{int}F4/80^{hi}$) frequency was unchanged (Fig. 1*B*, 1*C*). 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 (Supplementary Fig. 1). This increase in naturally-occurring Treg is thought to be due to DC-mediated Treg expansion (15). Pro-inflammatory cytokines are not expected to play a significant role in modulating the incidence or frequency of cell populations, as IL-1β, IL-4, IL-6, IL-10, IL-12p40, TNFα, and IFNγ were all nor detected in the serum of both PBS- and 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 naïve mice versus 441.1±26.15 pg/ml in Flt3L-treated mice; $p<0.05$).

We next sought to further characterize surface Ag expression on $CD11b^+Gr1^+$ cells expanded by Flt3L. Flt3L induced expansion of both $CD11b^+Ly6C^{int/hi}$ and $CD11b^+Ly6G^+$ cells (Fig. 1*E–G*). $CD11b^+Ly6C^{hi}$ cells expressed an intermediate level of F4/80 and were CD115 (M-CSF receptor)$^+$, consistent with surface Ag expression described for MDSC (Fig. 1*E*) (1). $CD11b^+Ly6C^{int}$ and $CD11b^+Ly6G^+$ cells were $F4/80^-$ and expressed only low levels of CD115 (Fig. 1*E*, 1*F*). Previously, 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. 1*H*). Moreover, both Flt3L-expanded $Ly6C^+$ and $Ly6G^+$ MDSC were significantly more potent suppressors than their counterparts from steady-state control mice (Fig. 1*H*). By contrast, $CD11c^+$ DC isolated from Flt3L-treated mice demonstrated increased allogeneic T cell stimulatory capacity (Fig. 1*I*). 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. 2*A–B*), consistent with earlier

reports using conditional STAT3 knockout mice (10). By contrast, expansion of $CD11b^+Gr1^+$ cells by Flt3L was augmented by STAT3 deletion (Fig. 2*A–C*). Chemical inhibition of STAT3 *in vivo* during Flt3L administration generated similar results (Supplementary 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. 2*D*). However, this did not occur when MDSC were isolated from mice treated with a STAT3 inhibitor and Flt3L (Supplementary Fig. 2*F*) potentially due to reversibility of STAT3 inhibition during *ex vivo* suppression. MDSC suppress T cell proliferation through several immunosuppressive enzymes, including arginase-1, inducible nitric oxide 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. 2*E*) and predominantly suppressed $CD4^+$ T cells without affecting Treg frequency (Supplementary Fig. 2*G–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 present study, $Gr1^+$ cells isolated from splenocytes of Flt3L-treated mice, but not control mice, prolonged fully MHC-mismatched cardiac allograft survival significantly in the absence of additional immunosuppression (Fig. 2*F*), thus demonstrating their *in vivo* suppressive function. Flt3L has been 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 for 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. Plus, 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard abbreviations used in this paper

DC	dendritic cell
Flt3L	Flt3 ligand
HO-1	heme oxygenase-1
MDSC	myeloid-derived suppressor cell
Treg	regulatory T cell

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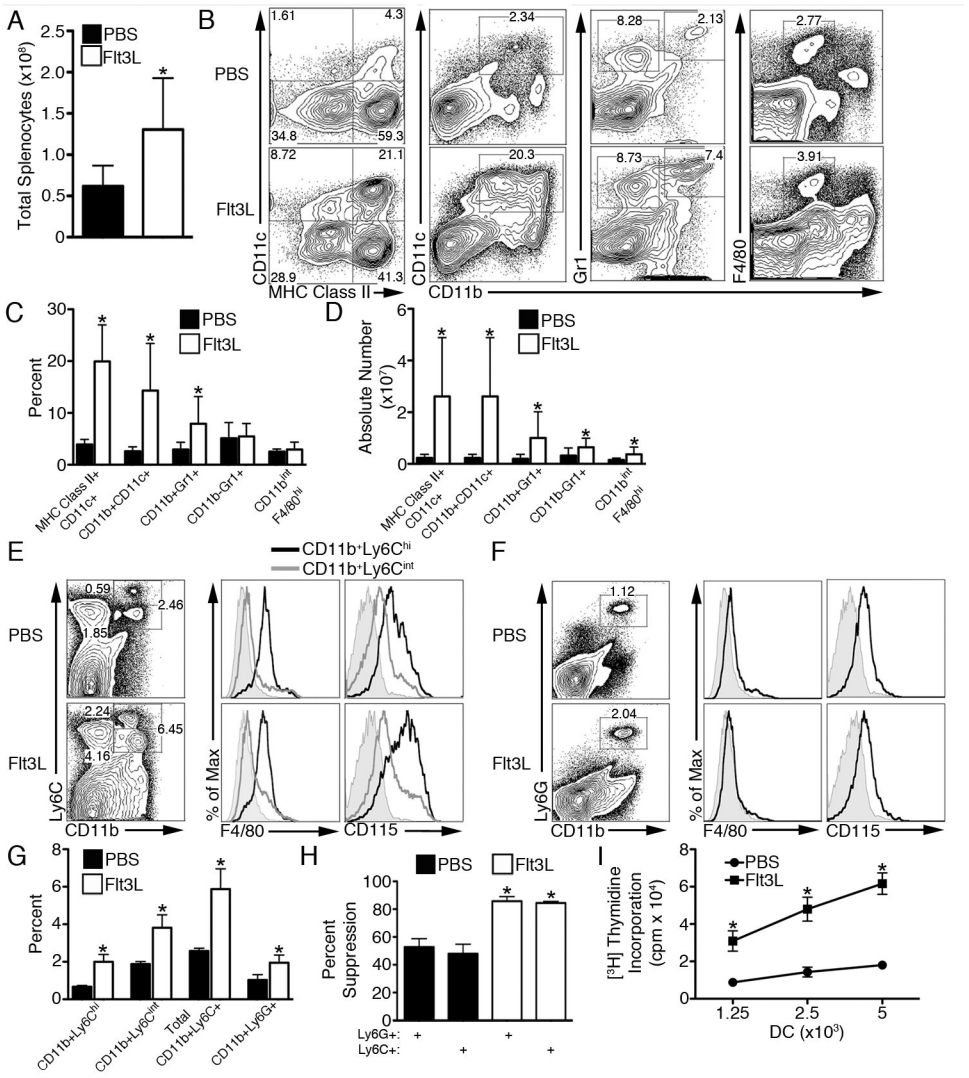

FIGURE 1.

Flt3L expands myeloid DC with augmented T cell stimulatory capacity but suppressive $CD11b^+Gr1^+$ cells. *A*, Total viable splenocytes from Flt3L-treated mice were enumerated using trypan blue exclusion. *B–C*, The frequency of splenic myeloid DC ($CD11c^+$MHC class II^+; $CD11b^+CD11c^+$), $CD11b^+Gr1^+$ cells, and macrophages ($CD11b^{int}F4/80^{hi}$) within $CD45^+$-gated cells was determined and (*D*) absolute number quantified. F4/80 and CD115 expression was determined on (*E*) $CD11b^+Ly6C^{int/hi}$ and (*F*) $CD11b^+Ly6G^+$ splenocytes and (*G*) their frequency quantified. *H*, BALB/c $Ly6C^+$ and $Ly6G^+$ splenocytes (2×10^5) were used as suppressors of B6 $CD3^+$ T cells (2×10^5) stimulated with Flt3L-mobilized BALB/c $CD11c^+$ DC (2×10^4). *I*, BALB/c $CD11c^+$ DC were used to stimulate B6 $CD3^+$ T cells (1×10^5). Data are representative of n≥2 experiments with n≥3 mice per group. *A–I*, * $p<0.05$.

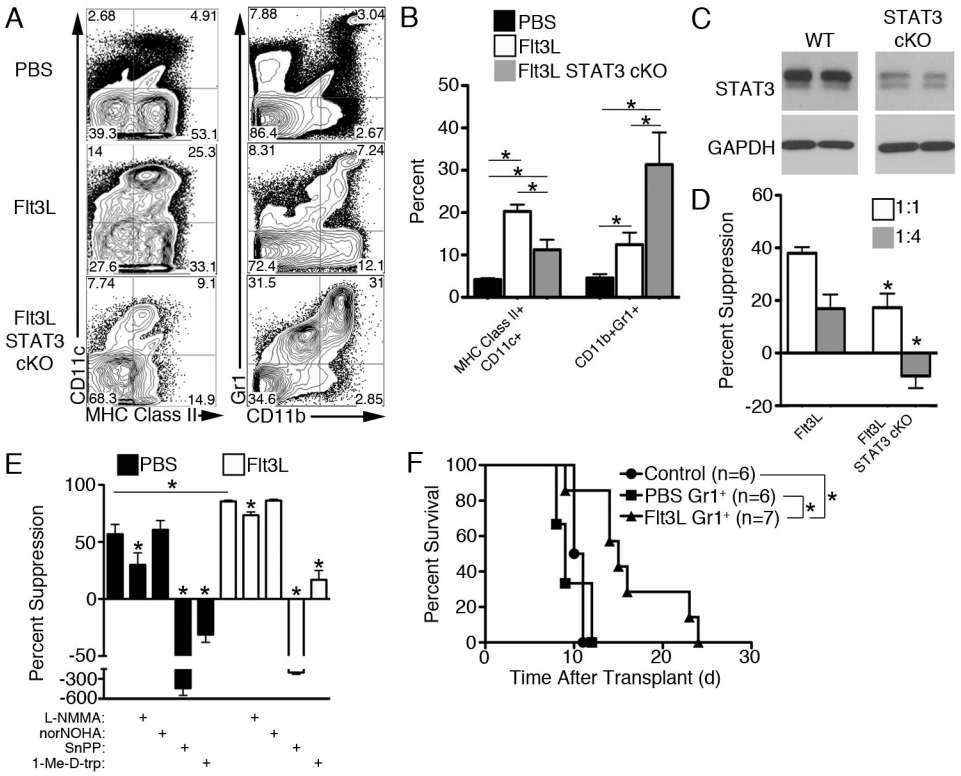

FIGURE 2.

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. Splenic DC and MDSC were identified by flow cytometry and (*B*) 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 were compared in suppression assays when splenic B6 Gr1$^+$ cells (1×10^5 or 0.25×10^5) were used as suppressors of B6 CD3$^+$ T cells (1×10^5) stimulated with BALB/c Flt3L-mobilized CD11c$^+$ DC (1.25×10^4). *E*, Splenic BALB/c Gr1$^+$ cells (1×10^5) were used as suppressors of BALB/c CD3$^+$ T cells (1×10^5) stimulated with B6 Flt3L-mobilized CD11c$^+$ DC (1.25×10^4). Inhibitors of nitric oxide synthase (N^G-Methyl-L-arginine; L-NMMA; 0.5 mM), arginase-1 (N^ω-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) were added at the start of co-culture where indicated. *F*, 5×10^6 BALB/c Gr1$^+$ cells were administered to BALB/c recipients i.v., 1 d before B6 heart transplant, and allograft survival was monitored. Data are representative of n=2 independent experiments with 6–7 total mice per group. *D*, * $p<0.05$ compared to Flt3L. *E*, * $p<0.05$ compared to PBS or Flt3L Gr1$^+$ cells in the absence of inhibitor, unless otherwise indicated.