Title

Fas ligand deficiency impairs tumor immunity by promoting an accumulation of monocytic myeloid derived suppressor cells

Running title: FasL affects MDSC numbers and subset distribution

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Abstract

The Fas receptor ligand FasL regulates immune cell levels by inducing apoptosis of Fas receptor-positive cells. Here we studied the impact of host FasL on tumor development in mice. Genetically targeting FasL in naive mice increased myeloid cell populations, but in marked contrast it reduced the levels of myeloid-derived suppressor cells (MDSC) in mice bearing LLC lung tumors. Analysis of the MDSC subset distribution revealed that FasL deficiency skewed cell populations toward the M-MDSC subset, which displays a highly immunosuppressive activity. Furthermore, tumor-bearing mice that were FasL-deficient displayed an enhanced proportion of tumor associated macrophages and T regulatory cells. Overall, the immunosuppressive environment produced by FasL targeting correlated with reduced survival of tumor-bearing mice. These results disclose a new role of FasL in modulating immunosuppressive cells.
Introduction

In various pathological situations, such as chronic inflammation and cancer, the differentiation of immature myeloid cells into mature granulocytes, macrophages and dendritic cells (DCs) is blocked, resulting in their expansion and conversion into potent immunosuppressive cells (MDSC, myeloid derived suppressive cells) (1). MDSCs consist of two major subsets, granulocytic (G-MDSC) and monocytic (M-MDSC) subpopulations that produce immune suppressive factors (arginase I, inducible nitric oxide synthase (iNOS) and cytokines), able to block effector T cell functions by different mechanisms, including the differentiation of naïve CD4 T cells into TH17 or Tregs populations (2-4). However, the mechanisms that regulate MDSC accumulation and subset distribution remain poorly understood. In addition to MDSCs, the stroma of many tumor types, both in mice models and human patients, contains macrophages (TAM, tumor associated macrophage) that are able to suppress immunity by different ways (5-7).

T-cell responses are regulated by a balance between stimulatory and inhibitory signals that are delivered by a set of immunoregulatory receptors and their ligands, such as PD-1 and its ligand PD-L1. High levels of PD-L1 were found in tumor environment (8, 9) and blockade of its interaction with PD-1 receptor results in tumor regression in patients with various cancer types (10). PD-1/PD-L1 interaction may exert its inhibitory effect on anti-tumor immunity, in part, by inducing CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) with suppressive function (11, 12). In steady state, Tregs are a specialized subset of CD4 T cells that have crucial role in maintaining peripheral tolerance. The absence of Tregs leads to autoimmunity (13), whereas increased Tregs promote tumor progression by interfering with tumor-specific T cells (14-16).
Fas-FasL pathway is an established homeostatic mediator of immune cell apoptosis (17-19). On tumor cells, the role of Fas-FasL pathway has been a matter of debate for a while. During tumor progression, Fas is frequently downregulated or cells are resistant to Fas-induced apoptosis raising the possibility that loss of Fas is a way of tumor evasion. However, complete loss of Fas is rarely seen in cancer patients (20). In addition, FasL or Fas deficient mice, despite the massive accumulation of immune cells, do not, in contrast to perforin deficient mice, develop spontaneous malignancies (17, 18, 21).

In the present study, we examined, in LLC-bearing mice (Lewis Lung Carcinoma, LLC), the impact of FasL on myeloid cell distribution and function. We show that the homeostasis of macrophages and DCs is FasL-independent and that FasL plays a key role in regulating the suppressive environment by controlling MDSC subsets, TAM, Tregs and as well as PD-1/PDL-1 expression levels.
Materials and Methods

Mice and tumors models

C57BL/6 Fasl−/− mice and control littermates (Fasl+/+), described previously (17), were housed in the animal facility at Gustave Roussy. Experimental animals were age- and sex-matched and used at 8–10 weeks of age. All experiments were approved by the institutional guidelines. Syngeneic tumor cell lines, LLC (Lewis Lung Carcinoma) and B16-F10 (melanoma cell line) were purchased from American Type Culture Collection (ATTC). E2 leukemic cells were produced by Dr Louache’s team according to Lavau et al (22). The cell lines B161F10 and LLC carcinoma cell lines were purchased from ATTC, amplified and kept in liquid nitrogen. We did not performed authentication of these cell lines. E2 cell line was provided by Dr Louache and was produced as indicated in the manuscript. We did not perform authentication. Retroviral pseudotypes were generated in 293T cells (from ATTC) transiently transfected with the retroviral vector MSCV/EGFP plasmid plus plasmids expressing MoMuLV env and gag/pol genes. Ly5.2 fetal liver (FL) cells were obtained from E16 pregnant mice and FL progenitors (Lineage negative) were enriched using the Midi MAX Lineage Depletion Kit (Miltenyi Biotech). For infections, virus particles were fixed onto 35-mm plates coated with Retronectin (Cambrex) before the addition of FL progenitors suspended in DMEM medium (Gibco-BRL) supplemented 50 ng/mL mSCF (PeproTech), 100 ng/mL FLT3-L (CellIdex Therapeutics, Inc., Needham, USA) and 10 ng/ml IL-3 (PeproTech). Infections were repeated after 24 hours. Transduced cells (10^6) supplemented with 2 × 10^5 congenic C57BL/6-Ly5.1 bone marrow (BM) cells were transplanted into irradiated (9,5 Gy) C57BL/6-Ly5.1 mice via the retro-orbital plexus. Tumor development was assessed by GFP and Ly5.2 expression on cell from BM at 8 weeks post transplantation. 80% of BM cells were positive for both
markers. Leukemic E2 cells were kept frozen in liquid nitrogen and, at time of use for experiments, they were tested for GFP expression. Mice were injected either intravenously (2 x 10^5 cells or 3 x 10^5 cells depending on tumor cell line) or subcutaneously (3 x 10^5 cells) as appropriately indicated in the text. Tumor growth was monitored by measuring two opposing diameters and volume was calculated as follow: (D x d^2) x 0.52. Tumors were analyzed when they reached an average volume of 327.5 ± 34.9 mm^3 and 728.8 ± 153.2 mm^3 for Fasl^+/+ and Fasl^−/− mice respectively within 15-23 days post tumor cells injection. GFP positive leukemic mice were analyzed at day 15-20-post injection. For induction of peritonitis, mice were given intra-peritoneal injection of 1 ml of 3% thioglycollate solution (Bio Merieux). At day 3-post injection, the recruited immune cells were removed from the peritoneum.

**Reagents, antibodies and Flow cytometry**

Recombinant murine GM-CSF was obtained from R&D Systems. Direct conjugated antibodies used for cell labeling were purchased from Affymetrix/eBiosciences: anti-F4/80 (BM8), anti-Ly6C (HK1.4), anti-Ly6G (1A8), anti-Gr-1 (RB6-8C5), anti-CD11b (M1/70), anti-CD4 ( GK1.5), anti-CD8 (53-6.7), anti-CD25 (PC61), anti-PD-1 (J43), anti PD-L1 (MIH5), anti-Foxp3, and Ki67 (clone SolA15). Fluorochrome-conjugated antibodies were used at the appropriate combinations and dilutions in staining buffer (PBS-0.5% BSA). All samples were treated with anti-CD16/32 (2.4G2) antibody to reduce Fc-receptor binding. For Foxp3 and Ki67, staining was performed according to the manufacturer’s instructions. Cell staining was examined by flow cytometry using the LSR II (BD). Data were analyzed by FACS DIVA 7.0 or Flow Jo 7.6.5 software. For functional studies, high-grade purified anti-CD3 (145-2C11), anti-CD28 (37.51), anti-PD-L1 (MIH5) antibody and its isotype control were used (Affymetrix/eBiosciences).
Cell isolation

Single cell suspensions were prepared from spleen by mechanical dissociation followed by removal of red blood cells with ACK buffer. Solid tumors were dissociated into small fragments and digested with collagenase XI and type IV bovine pancreatic DNase (Sigma-Aldrich) for 45 min at 37°C with gentle shaking. Red blood cells were removed by ACK buffer in single-cell suspensions. For peritoneal macrophages, thioglycollate-treated mice were injected with 5 ml of PBS to recover immune cells from peritoneum. Macrophages and MDSCs were, respectively, stained with conjugated CD11bF4/80 and CD11bGr-1 and sorted on a FACS Aria. This process yielded cell type suspension with purity >95%.

LLC conditioned (CM) medium preparation

LLC-derived conditioned medium was generated by culturing 2 x 10^5 cells/ml for 48h in DMEM/F12 supplemented with 10% FBS and penicillin/streptomycin. Supernatants were concentrated to a third of volume using 10 KDa molecular weight cut-off columns (Millipore).

Functional assays

For evaluation of T-cell proliferation, splenocytes from either naïve mice or LLC-bearing mice were cultured in the absence or the presence of MDSCs or macrophages at different ratios. Splenocytes were stimulated with either soluble anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) antibodies or coated anti-CD3 antibodies (5 μg/ml), as appropriately indicated in the text, for 72hrs at 37°C. Cell proliferation was measured by either MTT, a colorimetric assay for assessing cell viability and proliferation, or BrdU incorporation (BrdU Flow Kit, BD). For assessment of T cell
function, cytokine production was determined in the supernatants of activated splenocytes co-cultured with either MDSCs or macrophages. Cytokine levels were determined using the Mouse Th1/Th2 FlowCytomix Multiplex (Affymetrix/eBiosciences) according to the manufacturer’s instructions. Samples were quantified using the Flow Cytomix Pro 2.2 software (Affymetrix/eBiosciences). For PD-L1 blocking, MDSCs were treated with neutralizing PD-L1 antibody (5 μg/ml) or IgG control at 37°C for 2 hours prior to the addition of activated splenocytes.

**Arginase activity test and NO detection**

Arginase activity was measured in cell lysates (10⁶ cells) as previously described (23). For NO production, culture supernatants (100 μl) were mixed with equal volume of Greiss reagent and nitrite concentrations were determined by comparing the absorbance values of the test samples with a standard curve generated by a serial dilution of 0.250 mM sodium nitrite.

**Quantitative PCR**

Total RNA was extracted from the samples with TRIZOL solution (Invitrogen). 1 μg of total RNA was converted into cDNA by using TaqMan Reverse Transcription Reagent (Applied Biosystems) and mRNA levels were quantified by SYBR-GREEN qPCR method (Applied Biosystems) or TAQMAN qPCR method (Applied Biosystems). Relative expression was calculated by using the comparative Ct method (2−ΔΔCt).

**Statistical analysis**
Data were analyzed with GraphPad Prism. Nonparametric Mann-Whitney test was used to compare data between $Fasl^{+/+}$ and $Fasl^{+/−}$ mice. $P$ values $< 0.05$ were considered statistically significant.
Results

FasL controls tumor growth and mice survival

To investigate the role of FasL in tumor development, \( Fasl^{+/+} \) and \( Fasl^{-/-} \) mice were injected with LLC-tumor cell line and tumor growth and mice survival were followed over sixty-day period. As shown in figure 1A, the mortality of \( Fasl^{-/-} \) LLC-bearing mice was accelerated resulting in reduced survival rate compared to LLC-bearing \( Fasl^{+/+} \) mice. In addition, tumors grew faster in \( Fasl^{-/-} \) mice as indicated by the significantly increased tumor volumes in comparison to those of \( Fasl^{+/+} \) mice (Figure 1B).

FasL deficiency leads to a reduced MDSC population, but increased Treg population in tumor bearing-mice

Given the importance of FasL in the regulation of immune cells, we analyzed immune cells within the spleen of LLC-bearing mice at 21-23-day post injection. A significant increase in the total cell counts was observed in \( Fasl^{+/+} \) LLC-bearing mice when compared to naïve mice (Figure 2A). Strikingly, in \( Fasl^{-/-} \) LLC-bearing mice, the total spleen cell number was barely augmented when compared to \( Fasl^{-/-} \) naïve mice (Figure 2A).

In search of a cellular mechanism for the FasL-mediated tumor-suppressing effect, we investigated the immunosuppressive cells, MDSCs and Tregs. MDSCs are immature myeloid cells characterized by the expression of CD11b\(^+\)Gr-1\(^+\) cell markers. In the spleen of naïve \( Fasl^{+/+} \), the CD11b\(^+\)Gr-1\(^+\) population represented 3% of total leukocytes, whereas in FasL deficient mice, this population was significantly increased (Figure 2B-C). A similar significant increase was seen in the absolute number indicating that the homeostasis of this population is FasL-dependent. Also, the proportions of CD11b\(^+\)Gr-1\(^+\) population as well as CD11b\(^+\)Ly6C\(^+\)Gr-1\(^-\) monocytes
and CD11b+Ly6G+ neutrophils were significantly amplified in the blood of naïve Fasl−/− mice compared with controls (Supplemental Figure 1A).

In response to tumor development, the percentages as well as absolute cell number of CD11b+Gr-1+ cells were significantly increased in Fasl+/+ LLC-bearing mice compared to naïve Fasl+/+ mice (Figure 2B-C). By contrast, in Fasl−/− LLC-bearing mice, the small increase of CD11b+Gr-1+ cells, albeit significant in comparison to Fasl−/− naïve mice, remained remarkably low when compared with Fasl+/+ LLC-bearing mice (Figure 2B-C). Similar decrease of CD11b+Gr-1+ MDSCs was observed in LLC-tumor infiltrates of Fasl−/− mice (Supplemental Figure 1B). We checked whether macrophages (CD11b+F4/80+Gr-1−) or DCs could compensate for the decreased number of CD11b+Gr-1+ cells. In the spleen of naïve FasL deficient mice, number of macrophages (CD11b+F4/80+Gr-1−) was, as for DCs (19), significantly increased when compared to littermates (Supplemental Figure 1C). But, in LLC-bearing mice, we did not observe a significant difference in the numbers of either macrophages or DCs between the two genotypes (Supplemental Figure 1D). It is important to point out that the decrease of CD11b+Gr-1+ MDSCs cells is not restricted to LLC-tumor cell line. In B16-F10 melanoma-, as well as in E2 leukemic myeloid cells-, bearing mice, the proportion of CD11b+Gr-1+ MDSCs was reduced in the spleen and in B16-tumor infiltrates (Supplemental Figure 2 A-B).

To determine if the reduced MDSCs accumulation observed in Fasl−/− LLC-bearing mice was associated with an abnormal production of proinflammatory cytokines such as IFN-γ, GM-CSF and TNF-α, previously shown to be involved in MDSC accumulation (3), we measured their production in the sera and anti CD3/CD28-activated splenocytes. Interestingly, the production of IFN-γ, GM-CSF and TNF-α was similar in Fasl+/+ and Fasl−/− mice (Figure 2D). Thus, in the absence of FasL, the
pro-inflammatory conditions generated by tumor injection could not support the MDSCs accumulation, suggesting a unique role for FasL.

In addition to MDSCs, Tregs are well known immunosuppressive population of CD4 T cells in tumor environment. We found that, by contrast to MDSCs, Tregs were significantly increased in Fasl<sup>-/-</sup> LLC-bearing mice. As shown in Figure 2E, the increase of CD25<sup>High</sup>FoxP3<sup>+</sup> proportion in Fast<sup>-/-</sup> mice, albeit weak, was significant when compared with control mice. The increase of Treg cells is induced by tumor environment, as naïve Fast<sup>-/-</sup> mice exhibited similar proportion of CD25<sup>High</sup>FoxP3<sup>+</sup> as control mice (Figure 2E).

**FasL deficiency skews MDSC population towards M-MDSC subset**

MDSCs are divided into monocytic (M-MDSCs, Gr-1<sup>low</sup>) and granulocytic (G-MDSCs, Gr-1<sup>high</sup>) subsets based on the differential expression of Ly6C and Ly6G antigens respectively, along with the brightness of Gr-1 expression. In addition, G-MDSCs subset is the prevalent population of MDSCs in different tumor models (24, 25). In the spleen of Fasl<sup>+/+</sup> LLC-bearing mice, the proportion of Gr-1<sup>high</sup>Ly6G<sup>+</sup> G-MDSC subset was higher than Gr-1<sup>low</sup>Ly6C<sup>+</sup> M-MDSC proportion (Figure 3A). This leads to the expected ratio of 60% of G-MDSCs within the CD11b<sup>+</sup>Gr-1<sup>+</sup> population (Figure 3B-C). Strikingly, in Fast<sup>-/-</sup> LLC-bearing mice, although both MDSC subsets were reduced, this reduction was significantly pronounced for G-MDSC subset leading to an inverted ratio of MDSC subsets as illustrated in Figure 3A-C. The same MDSC subset distribution was observed in tumor infiltrates from Fasl<sup>-/-</sup> mice (Supplemental Figure 3A-B).

**FasL deficient MDSCs strongly suppress T cell responses**
We next evaluated the capacity of sorted CD11b+Gr-1+ MDSCs from the spleen of LLC-bearing mice to suppress *in vitro* the proliferation of splenocytes from normal mice stimulated with anti-CD3/CD28 antibodies. We found that sorted MDSCs from either *Fasl*+/+ or *Fasl*−/− mice were devoid of suppressive activity, as shown by the absence of inhibitory effect on T cell proliferation (Figure 4A). However, when they were treated for four days with GM-CSF, both FasL positive and FasL negative MDSCs acquired a potent suppressive activity as shown by the inhibition of proliferative response of anti-CD3/CD28-activated splenocytes as well as IFN-γ production (Figure 4A and C) and by the production of IL-10 and TNF-α (Figure 4B). Interestingly, MDSCs from *Fast*−/− mice produced higher amount of IL-10 and TNF-α and much lower level of IFN-γ compared with control MDSCs, but they displayed similar arginase activity as MDSCs from *FasL*+/+ mice (Supplemental Figure 4). Furthermore, the production of IFN-γ by activated T cells was at least two-fold lower in the presence of FasL deficient MDSCs as compared to FasL positive MDSCs (Figure 4C); this is in line with the pronounced inhibition of T-cell proliferation (Figure 4A). This suppressive activity was amplified when MDSCs were cultured with GM-CSF and conditioned medium from LLC cells (CM) as shown by a further reduced amount of IFN-γ and decreased T cell proliferation (Figure 4A and C) indicating that additional tumor-derived factors emphasize the immunosuppressive function of MDSCs. Indeed, sorted MDSCs from tumor infiltrates of *Fasl*+/+ or *Fasl*−/− mice were directly able to significantly suppress proliferative response of activated T cells (Figure 4D).

**The PD1/PD-L1 pathway contributes to the suppressive activity in FasL deficient mice**
Both immune and tumor cells express PD-L1 and its interaction with PD-1 receptor represents a major obstacle to antitumor immunity. We found significantly higher level of PD-L1 expression by splenocytes from Fasl−/− mice compared with controls (Figure 5A). Interestingly enough, when PD-L1 expression was gated in MDCS population, we found a significant increased percentage of PD-L1-expressing M-MDSC subset in Fasl−/− mice relative to control mice. While similar percentage of PD-L1 positive G-MDSC subsets was observed in both genotypes (Figure 5B). In the same way, we compared PD-1 expression on CD8 T cells and found a significant higher percentage of PD-1-expressing CD8 T cells in Fasl−/− mice compared with control mice, while similar numbers of CD8 T cells were observed (Figure 5C). To test the functional consequence of PD-L1 and PD-1 overexpression in MDSC-mediated T cell suppression, PD-L1 expression was blocked on ex vivo MDSCs by using neutralizing anti-PD-L1 monoclonal antibody. Interestingly, PD-L1 blockade on MDSCs, abrogated the suppressive activity of MDSCs. As shown in figure 5D, the ability of FasL positive and FasL negative GM-CSF/CM-treated MDSCs to inhibit T cell proliferation was removed after blocking with anti-PD-L1, but not with IgG control. Thus, PD-L1 expression on MDSCs is involved in mediating the suppressive action of MDSCs, at least in part, as we were not able to completely restore the proliferative T cell response after PD-L1 blockade.

**FasL deficiency results in macrophage accumulation in the tumor microenvironment**

A study from Movahedi et al. suggested that the M-MDSC subset might be a progenitor of TAM (26). In Fasl−/− mice, we observed a prevalence of M-MDSCs. We, therefore, investigated TAM (CD11b+F4/80+Ly6C+/−Gr-1+) in established subcutaneous LLC-tumor from Fasl−/+ and Fasl−/− mice. Tumors from Fasl−/− were
significantly enriched with CD11b+F4/80+Ly6C+/−Gr-1− macrophages compared with those from Fasl+/+ mice (Figure 6A). We therefore, evaluated the potential suppressive function of sorted CD11b+F4/80+ macrophages by different ways. Gene expression analysis showed that FasL deficient macrophages over expressed IL-6 and IL-10 immunosuppressor genes and inducible nitric oxyde synthase (iNOS) compared with FasL proficient macrophages. The IL-10 gene over expression was correlated with enhanced production of IL-10 and reduced IFN-γ production (Figure 6B-C). While, nitrite production (NO) and arginase activity levels were similar in macrophages from both genotypes (Figure 6D). Also, inhibition of T cell responses was similar with macrophages from both genotypes as demonstrated by the low levels of the proliferative response as well as IFN-γ and GM-CSF production of anti-CD3-activated splenocytes (Figure 6E-F). It is worth noting that the accumulation of suppressive macrophages was not restricted to tumor microenvironment, as thioglycollate-induced inflammatory peritonitis resulted in a significant increased proportion of suppressive macrophages in the peritoneum of Fasl−/− mice compared with control mice (Supplemental Figure 5A-B). All together, these results suggested that in inflammatory environment FasL might control suppressive cells and impact their suppressive activity.
Discussion

FasL, a cytokine with apoptotic and proinflammatory functions, is implicated in the development of autoimmunity. However, the degree to which FasL contributes to malignancies remains poorly explored, since FasL deficient mice do not develop over age spontaneous tumors (17). In the present work, we showed that, FasL deficiency generated an immunosuppressive environment, as M-MDSCs, Tregs and PD-1/PD-L1 expression were significantly enhanced. This phenotype led to an accelerated tumor growth and reduced survival rate of the mice.

FasL signaling is crucial for maintaining the homeostasis of myeloid cells including the CD11b+Gr-1+ immature cells (19). In cancers, the differentiation of immature CD11b+Gr-1+ myeloid cells is blocked leading to their accumulation and conversion into potent immunosuppressive cells, therefore, promoting tumor growth. The accumulation of MDSCs could be attributed to defect in death signals; an excess of pro-inflammatory factors produced by tumors, host cells or both or to aberrant regulation of myelopoiesis. In the current work, we showed that FasL-mediated cell death does not interfere with the regulation of MDSCs level. In Fasl−/− LLC-bearing mice, as well as in mice bearing B16-F10 melanoma or E2-myeloid leukemia cells, MDSCs, in both lymphoid organs and tumor sites, were not over numbered compared with control mice. These results indicate that other death signaling pathways regulate MDSC accumulation, which is in line with a recent report demonstrating the implication of intrinsic apoptotic pathway in MDSCs accumulation (27). However, our results contrast with study by Sinha et al. suggesting that FasL-mediated apoptosis regulates MDSC level in LLC-bearing gld mice (28) The discrepancy could be attributed to the use of tumor models with different genetic background (C57BL/6 versus Balb/c mice).
MDSCs were the cells primary affected by FasL deficiency, as their number was reduced and population structure was modified. The reduced number of MDSCs in Fast<sup>-/-</sup> mice was not associated with a defect of proinflammatory cytokine production, which suggests that FasL might control the differentiation of immature myeloid cells into macrophages or DCs. Indeed, tumor infiltrates from Fast<sup>-/-</sup> mice were significantly enriched with macrophages compared to control mice. Further investigations are needed to identify how FasL impacts MDSC maturation. Nevertheless, our results are in agreement with reports demonstrating that another member of the TNF super family, TNF-α, affects MDSC differentiation and accumulation in chronic inflammation (29). The mechanisms that regulate MDSC subsets are still poorly known. Recently, Youn et al. showed that G-MDSCs were derived from the highly proliferative M-MDSC pool that acquired morphological and phenotypical features of G-MDSCs (24). Here, we showed that in the absence of FasL, MDSC population was skewed toward M-MDSC subset (Fig 3) with enhanced suppressive activity. These data raise the possibility that the development and/or survival of G-MDSC subset are dependent on FasL signaling pathway. Alternatively, and according to the work by Youn <i>et al.</i>, FasL might control the process of M-MDSC conversion into G-MDSC. Furthermore, our results provide the evidence that suppression is principally found in the monocytic component of the MDSC pool, which is in line with a very recent report by Haverkamp <i>et al.</i> showing that selective loss of G-MDSC subset did not alter tumor incidence (30). We believe however that the role of FasL in MDSC compartment requires further investigations to decipher whether the impact of FasL is intrinsic or extrinsic and how it controls MDSC subset regulation in tumor-induced inflammation.

Our data showed that FasL impacts other immunosuppressive elements, such as Tregs and PD-1/PD-L1 axis. In the absence of FasL, PD-L1 expression was
increased on myeloid and non-myeloid cells (data not shown) and blocking PD-L1 on ex vivo MDSCs enhances MDSC-mediated T cell proliferation, which is in correlation with recent studies (9, 31). In addition, MDSCs are not the only source of PD-L1 to turns down T cell responses in vivo. PD-L1 expressed on APC has been shown to play a role in the induction and maintenance of peripheral suppressive Tregs (32).

We found an increased proportion of Tregs in the absence of FasL that can not be attributed to a defect of FasL-mediated T-cell homeostasis, as this process is FasL-independent (18), but Bcl-2 dependent (33). Thus, we could conceive that the increase of PD-L1 level on Gr-1 negative myeloid cell might contribute to the induction of peripheral Tregs. In tumor environment, up regulation of PD-L1 expression depends on proinflammatory cytokines, mainly IFN-γ, and on hypoxia signaling pathway (9, 34). It will be interesting to examine how FasL impacts PDL-1 promoter regulation by investigating molecules such, NF-kb, JAK/STAT and IRF known to bind to PD-L1 promoter.

MDSCs and PDL-1 levels are enhanced in patients with lung cancer and other cancer types, including hematological malignancies, with a role in disease progression and/or drug resistance. Different drugs and immune checkpoint blockers, such as sunitinib and anti-PDL-1 antibody that potentially interfere with MDSC and PDL-1 activities respectively, are under investigation or already available in lung cancer and other malignancies (35-38). As our results provide a relationship between FasL-signaling pathway and the developing immunosuppressive elements, MDSCs and PDL-1, targeting FasL may potentially represents an interesting opportunity to enhance these therapeutic strategies in cancer and possibly in other pathologic conditions.
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References


Figure legends

Figure 1. FasL affects tumor growth and mice survival.

(A) Survival rate of Fast+/+ and Fast−/− mice intravenously injected by 2 x 10^5 LLC cells. 
(B) Tumor growth in Fast+/+ and Fast−/− mice subcutaneously injected by 3 x 10^5 LLC tumor cells. n = 10 per group; *P < 0.04.

Figure 2. MDSCs are reduced in LLC-bearing Fast−/− mice.

CD11b+Gr-1+ population was analyzed in the spleen from 7 week-old naïve and from tumors-bearing mice at day 21-23-post subcutaneous injection of 3 x10^5 LLC-tumor cells. (A) Absolute cells numbers of splenocytes from naïve and from LLC-bearing mice. (B-C) Percentages and absolute numbers of CD11b+Gr-1+ cells in the spleen from naïve and LLC-bearing Fast+/+ and Fast−/− mice. (D) Concentrations of IFN-γ, GM-CSF and TNF-α in the sera and supernatants of anti-CD3/CD28-activated splenocytes from LLC-bearing mice. (E) Percentages of splenic CD4 T cells and of CD4CD25^{High}Foxp3+ Treg cells from LLC-bearing and naïve mice. n = 6-9 per group for naïve mice. n = 11-13 for MDSCs and n = 9-12 for Tregs per group of LLC-bearing mice; * P ≤ 0.03; ** P ≤ 0.004; ***P ≤ 0.0005.

Figure 3. FasL deficient CD11b+Gr-1+ MDSCs are enriched with M-MDSC subset.

MDSC subsets were analyzed in the spleen from LLC-bearing mice at day 21-23 post subcutaneous injections of 3 x10^5 LLC-tumor cells. (A) Percentages of MDSCs in the spleen and of MDSC subsets within the CD11b+Gr-1+ population. (B) Representative histogram of Gr-1 expression and (C) Ratio of MDSC subsets gated in CD11b+Gr-1+ MDSC population for all experimented animals. n = 9, *P ≤ 0.04, ***P ≤ 0.0006
Figure 4. Splenic MDSCs suppress T cell responses.

CD11b^Gr-1^ MDSC population was isolated from the spleen and tumor infiltrates of \textit{Fast}^+/+ and \textit{Fast}^-/- mice at 21-23 days post subcutaneous injection of 3 x10^5 LLC-tumor cells. Splenic MDSCs (Spl MDSC) were treated with GM-CSF (10 ng/ml) with or without CM for 4 days and anti-CD3/CD28-activated splenocytes from naïve mice were added at 1:1 ratio for additional three days. (A) Inhibition of T-cell proliferative response by splenic MDSCs, *P \leq 0.04. (B) Production of cytokines by splenic MDSCs treated with GM-CSF and CM. (C) IFN-γ production by activated T cells in the absence and the presence of treated spleninc MDSCs. (D) Inhibition of T cell proliferation by sorted MDSCs from tumor infiltrates (Inf MDSC).

Figure 5. PD-L1-mediated immunosuppression of MDSCs.

Expression of PD-L1 and PD-1 was determined in splenocytes at 21-23 days post LLC-tumor cell injection (3 x10^5 cells). (A) Representative histogram of PD-L1 expression in total splenocytes. (B) PD-L1 expression gated in Gr-1^LowCD11b^ MDSCs and in Gr-1^HighCD11b^ G-MDSCs. (C) Number and percentages of CD8 T cells and PD-1-expressing CD8 T cells. (D) Blocking of PD-L1 pathway enhances CD8 T cell proliferation. GM-CSF/CM-treated MDSCs were incubated for 2 hours at 37°C with 5 μg/ml control antibody (IgG) or neutralizing PD-L1 antibody and cocultured at 1:1 ratio with anti-CD3/CD28 activated CD8 T cells from tumor bearing mice for three days. T cell proliferation was measured by Ki67 labeling. n = 8 per group; *P \leq 0.04, P \leq 0.005, **P <0.0001

Figure 6. Suppressive macrophages accumulate in tumor site of \textit{Fast}^-/- mice.
Intra-tumoral macrophages were characterized at day 21-23-post subcutaneous injection of 3 x10^5 LLC-tumor cells. (A) Percentages of CD11b^+F4/80^+Gr-1^- macrophages in tumor infiltrates, *P ≤ 0.03. (B) TNF-α, IL-6, IL-10 and iNOS gene expression by macrophages. (C) Spontaneous production of IL-10 and IFN-γ by sorted macrophages. (D) NO production and enzymatic arginase of macrophages. NO production was determined in the presence of anti-CD3 activated splenocytes at 1:1 ratio; arginase activity was measured in the lysates (10^6 cells) of directly sorted macrophages. (E-F) macrophage-induced inhibition of (E) anti-CD3 activated T cell proliferative response and (E) IFN-γ and GM-CSF production.
Figure 1
Figure 2

A

B

C

D

E

Activated splenocytes

[Graphs showing statistical analysis and data visualization]
Figure 3
Figure 4
Figure 5
Figure 6

A

% CD11b+F4/80+ macrophages

Fast+/+ Fast+/

B

Fold-change relative to control

TNF-α IL-6 IL-10 INOS

C

IL-10 (pg/ml)

Fast+/+ Fast+/

IFN-γ (pg/ml)

Fast+/+ Fast+/

NO (mM)

Fast+/+ Fast+/

Urea per 10^6 cells (mM)

Fast+/+ Fast+/

D

E

% T cell proliferation

Fast+/+ Fast+/

Splenocytes : macrophages

1 : 0 1 : 1

F

IFN-γ (pg/ml)

Fast+/+ Fast+/

1 : 0 1 : 1 1 : 1

Splenocytes : macrophages

1 : 0 1 : 1

GM-CSF (pg/ml)

Fast+/+ Fast+/

1 : 0 1 : 1
Fas ligand deficiency impairs tumor immunity by promoting an accumulation of monocytic myeloid derived suppressor cells

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