Published in final edited form as:

Eur J Immunol. 2014 August ; 44(8): 2457–2467. doi:10.1002/eji.201444497.

IFN- γ regulates survival and function of tumor-induced CD11b⁺Gr-1^{high} myeloid derived suppressor cells by modulating the anti-apoptotic molecule Bcl2a1

José Medina-Echeverez^{1,*}, Lydia A. Haile^{1,*}, Fei Zhao¹, Jaba Gamrekashvili¹, Chi Ma¹, Jean-Yves Métais², Cynthia E. Dunbar², Veena Kapoor³, Michael P. Manns⁴, Firouzeh Korangy¹, and Tim F. Greten¹

¹GI-Malignancy Section, Thoracic and GI-Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

²Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA

³Experimental Transplantation and Immunology Branch, NIH, Bethesda, USA

⁴Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany

Abstract

Myeloid derived suppressor cells (MDSCs) play a critical role in suppression of immune responses in cancer and inflammation. Here, we describe how regulation of Bcl2a1 by cytokines controls the suppressor function of CD11b⁺Gr-1^{high} granulocytic MDSCs. Co-culture of CD11b⁺Gr-1^{high} granulocytic MDSCs with antigen-stimulated T cells and simultaneous blockade of IFN- γ by the use of anti-IFN- γ blocking antibody, IFN- γ ^{-/-} effector T cells, IFN- γ R^{-/-} MDSCs or STAT1^{-/-} MDSCs led to up-regulation of Bcl2a1 in CD11b⁺Gr-1^{high} cells, improved survival and enhanced their suppressor function. Molecular studies revealed that GM-CSF released by antigen-stimulated CD8⁺ T cells induced Bcl2a1 up-regulation, which was repressed in the presence of IFN- γ by a direct interaction of phosphorylated STAT-1 with the Bcl2a1 promotor. Bcl2a1 overexpressing granulocytic MDSCs demonstrated prolonged survival and enhanced suppressor function in vitro. Our data suggest that IFN- γ /STAT1-dependent regulation of Bcl2a1 regulates survival and thereby suppressor function of granulocytic MDSCs.

Keywords

G-MDSC; IFN- γ ; GM-CSF; Bcl2a1; vaccine; immunotherapy

Correspondence should be sent to: Tim F. Greten, M.D., National Cancer Institute, Building 10 Rm 12N226, 9000 Rockville Pike, Bethesda MD 20892, USA, Tel: +1-301 451 4723, Fax +1-301 480 8780, tim.greten@nih.gov.

*These two authors contributed equally to the work

Conflict of interest

The authors declare no commercial or financial conflict of interest.

Introduction

Myeloid derived suppressor cells (MDSCs) have important regulatory functions in chronic inflammation and cancer [1]. They are known for their diversity and heterogeneity both in cancer patients and different murine tumor models [2]. In mice, MDSCs are composed of two major subsets namely monocytic Gr-1^{dull/int.} (CD11b⁺Ly6C^{high}Ly6G⁻) and granulocytic Gr-1^{high} (CD11b⁺Ly6C^{low}Ly6G^{high}) cells. Granulocytic MDSCs (G-MDSCs) possess a higher potential of *in vivo* expansion than monocytic MDSCs whereas monocytic MDSCs (M-MDSCs) have a higher suppressive activity in different tumor models [3, 4].

Previous reports have suggested that granulocytic and monocytic MDSC subsets use different effector molecules and signaling pathways in suppressing antigen-specific immune responses. CD11b⁺Ly6C^{high}Ly6G⁻ M-MDSCs suppress antigen-specific responses utilizing NO in an IFN- γ -dependent manner [4]. On the other hand, CD11b⁺Ly6C^{low}Ly6G^{high} G-MDSCs produce higher levels of ROS whereby ROS scavengers completely reverse their inhibitory role [4, 5]. A number of cytokines and transcription factors were shown to modulate the differentiation, expansion and activation of MDSCs [6–12]. Among these, IFN- γ , a pleiotropic cytokine expressed by T cells, dendritic cells and NK cells has been shown to modulate immunosuppressive function of monocytic MDSCs [13–15]. The effect of IFN- γ was demonstrated to be due to its ability to induce NOS2 and subsequent NO release [15, 16]. A recent study revealed that *in vivo* treatment of mice with a combination of LPS and IFN- γ enhances the expansion and suppressor activity of both MDSC subsets and blocks development of bone-marrow precursors into dendritic cells [17, 18].

Here, we present a novel regulatory mechanism for the suppressor function of G-MDSCs. Upon stimulation of T cells with antigen, T cells release simultaneously GM-CSF and IFN- γ . These two cytokines have opposing effects on Bcl2a1 expression in G-MDSCs thereby controlling cell survival and consecutively granulocytic MDSC suppressor function. We demonstrate that GM-CSF induces up-regulation of the anti-apoptotic protein Bcl2a1, while IFN- γ blocks STAT-1 dependent Bcl2a1 interaction. In addition, prolonged G-MDSC survival in the absence of IFN- γ enhances their suppressor function. Finally, Bcl2a1 overexpression in G-MDSCs enhances their suppressive potential *in vitro* and *in vivo*. In summary, our data provides evidence that the local cytokine environment can dictate MDSC function.

Results

IFN- γ has a distinct role in the suppressive ability of MDSC subsets

FACS-sorted M-MDSCs from spleens of CT-26 tumor bearing mice strongly suppressed antigen-specific T-cell proliferation compared to G-MDSCs (Figure 1A). Interferon gamma (IFN- γ) has been described to enhance the immunosuppressive function of monocytic MDSCs [4]. In order to understand the regulation of the suppressor activity of G-MDSCs, MDSC subsets were co-cultured with antigen-specific T cells in the presence of an IFN- γ neutralizing antibody. G-MDSCs efficiently suppressed T-cell responses rather in the presence than in the absence of anti-IFN- γ antibody, indicating an inhibitory role of IFN- γ on their function (Figure 1B). In contrast and as expected, M-MDSCs lost their suppressor

ability when IFN- γ or iNOS was blocked (Figure 1C). Neither addition of the NO synthase inhibitor NG-monomethyl-L-arginine (L-NMMA) nor the arginase inhibitor NG-monomethyl-L-arginine (nor-NOHA) to G-MDSCs had an effect on their suppressor function (Figure 1B) indicating that suppression by G-MDSCs was independent of NOS2 and arginase. Furthermore, NO was only detected in co-cultures of T cells with monocytic but not granulocytic MDSCs (data not shown). Hence, IFN- γ has opposing effects on the ability of granulocytic and monocytic MDSCs to suppress T-cell proliferation *in vitro*.

IFN- γ reduces the suppressive effect of G-MDSC

IFN- $\gamma^{-/-}$ antigen-specific T cells were used in subsequent experiments to analyze the IFN- γ -dependent regulation of G-MDSC function in more detail. Interestingly, the suppressor ability of tumor-induced G-MDSCs was significantly higher than M-MDSCs when co-cultured with CL-4 TCR IFN- $\gamma^{-/-}$ T cells (Figure 2A), as previously seen with IFN- γ blockade. This effect was reversed by the addition of recombinant IFN- γ to co-cultures (Figure 2A). Exogenous IFN- γ had minimal effects in suppressor function of wild type G-MDSCs derived from CT-26 tumor bearing mice (Figure 2B). In contrast, suppressor activity was significantly enhanced when IFN- γ was added in a dose dependent manner to M-MDSCs (Figure 2C), suggesting that IFN- γ promotes immunosuppression driven by monocytic MDSCs.

To exclude the indirect effect of IFN- γ in the assay system chosen, we next examined MDSCs derived from IFN- $\gamma R^{-/-}$ mice. As shown in Figure 2D, splenic IFN- $\gamma R^{-/-}$ G-MDSCs from EL-4 tumor bearing littermates exhibited a profound and significant suppressive activity. However, IFN- $\gamma R^{-/-}$ M-MDSCs failed to inhibit OVA-mediated T-cell proliferation. In summary, G-MDSCs become potent immune suppressors not only when IFN- γ is exogenously blocked or not produced by antigen-specific T cells, but also when G-MDSCs lacked the IFN- γ receptor.

Bcl2a1 regulates the suppressive function of G-MDSC

To understand how granulocytic MDSCs become potent immunosuppressive cells in the absence of IFN- γ , we performed a gene expression analysis on G-MDSCs cultured alone versus G-MDSCs resorted after co-culture with IFN- $\gamma^{-/-}$ HA-specific T cells (Table 1) (GEO database accession no. GSE32000). Several cell death associated genes were up-regulated in G-MDSCs in the absence of IFN- γ producing CL-4 T cells. Among them Bcl2a1 expression was 52- fold increased. Other differentially expressed genes included SOCS1, CD14 and the transcription factor CEBP β , which has been shown to be a critical factor for MDSC-mediated immune suppression [7]. Differential Bcl2a1 expression was confirmed by quantitative PCR. IFN- $\gamma^{+/+}$ CL-4 TCR and/or IFN- $\gamma^{-/-}$ CL-4 TCR splenocytes were incubated with CT-26 splenic G-MDSCs and quantitative real-time PCR analysis was performed after 12 and 48 hours. Bcl2a1 expression was significantly increased in granulocytic MDSCs co-cultured for 12 hours with IFN- $\gamma^{-/-}$ CL-4 TCR splenocytes than in G-MDSCs co-cultured with IFN- $\gamma^{+/+}$ CL-4 TCR cells (Figure 3A) and remained elevated up to 48 hours (Figure 3B). Similar results were obtained when Bcl2a1 expression was analyzed in G-MDSCs co-cultured in the presence of anti-IFN- γ neutralizing antibody

(Figure 3C). With the exception of Mcl-1 other apoptosis-related genes were not substantially affected (Figures 3D and 3E).

GM-CSF induces up-regulation of Bcl2a1 in G-MDSC

As shown in Figures 3A and 3B, Bcl2a1 levels were similar in G-MDSCs alone and in the presence of antigen-stimulated T cells, but increased when G-MDSCs were incubated with IFN- $\gamma^{-/-}$ CL-4 cells. This fact suggests that antigen-stimulated T cells may provide a signal for up-regulation of Bcl2a1 in G-MDSCs, which is counter-regulated by the expression of IFN- γ . GM-CSF is an important growth factor for the accumulation of MDSCs [1], which has been shown to up-regulate Bcl2a1 expression [19] and is expressed by T cells upon antigen stimulation. Therefore, we studied the specific effect of GM-CSF in this setting. Incubation of G-MDSCs with GM-CSF led to a dose dependent increase in Bcl2a1 expression (Figure 4A). Addition of IFN- γ expressing T cells to splenic CT-26 G-MDSCs abrogated Bcl2a1 expression similar to IFN- γ when added to G-MDSCs incubated with IFN- $\gamma^{-/-}$ CL-4 cells (Figure 4B). To examine the relevance of this finding for the survival of G-MDSCs we quantified the percentage of Annexin-V⁷-AAD⁻ granulocytic MDSCs after co-culture with IFN- $\gamma^{+/+}$ CL-4 or IFN- $\gamma^{-/-}$ CL-4 TCR splenocytes. A higher percentage of alive Annexin-V/7-AAD double negative CD11b⁺Gr-1^{high} cells was detected in co-cultures with IFN- $\gamma^{-/-}$ CL-4 TCR splenocytes than in co-cultures with IFN- $\gamma^{+/+}$ CL-4 TCR splenocytes (Figure 4C). Addition of exogenous IFN- γ to IFN- $\gamma^{-/-}$ effector T cells and G-MDSCs co-cultures resulted in a dose-dependent decrease in the percentage of viable Annexin-V/7-AAD double negative CD11b⁺Gr-1^{high} cells (Figure 4D). Furthermore, increased viability of G-MDSCs was also observed after incubation with GM-CSF (Supplementary Figure 2A). In contrast, GM-CSF blockade or addition of recombinant IFN- γ to antigen-stimulated T cell/G-MDSC co-cultures substantially decreased G-MDSC survival (Supporting Information Fig.2B). Finally, we tested the effect of other cytokines implicated in MDSC biology such as TNF- α [20] and IL- β [21] on Bcl2a1 expression but did not observe any effect alone or in combination (Supporting Information Fig.2C). Thus, IFN- γ and GM-CSF affects the functional status and regulates the survival of tumor – induced G-MDSCs.

Bcl2a1 down-regulation in granulocytic MDSC requires STAT1

IFN- γ triggers phosphorylation of STAT1 in order to activate or repress target gene transcription [22]. Therefore, we analyzed the phosphorylation status of STAT1 in monocytic and granulocytic MDSCs by FACS after treatment with IFN- γ . We observed phosphorylated STAT1 in both subsets when exposed to IFN- γ (Figure 5A). However, IFN- γ did not induce additional phosphorylation of STAT3, a main factor for mouse and human MDSC expansion. [12]. Based on this observation, we proposed that IFN- γ induces STAT1 phosphorylation and therefore Bcl2a1 down-regulation in G-MDSCs when incubated with antigen-stimulated T cells. Hence, we studied whether STAT1 regulates the expression of Bcl2a1 in G-MDSCs using STAT1^{-/-} mice bearing 344SQ subcutaneous tumors. First, we quantified the frequency of MDSC subsets in tumor bearing WT and STAT1^{-/-} mice and observed no significant differences in this tumor model (Figure 5B). As expected, STAT1-deficient M-MDSC cells were unable to inhibit T-cell proliferation compared to STAT1^{+/+} M-MDSCs. However, STAT1^{-/-} G-MDSCs demonstrated a significantly stronger

suppressor activity than G-MDSCs from STAT1^{+/+} littermate controls (Figure 5C). Finally, Bcl2a1 up-regulation was detected in STAT1^{-/-} G-MDSCs from 344SQ tumor bearing mice co-cultured with antigen-stimulated T cells (Figure 5D).

IFN- γ controls Bcl2a1 expression through STAT1

Next we tested the effects of GM-CSF and IFN- γ on Bcl2a1 expression. As expected GM-CSF increased Bcl2a1 mRNA expression in RAW264.7 cells, which was reversed by addition of IFN- γ (Figure 6A) similar to our previous observation in G-MDSCs incubated with antigen stimulated T cells in the presence of anti-IFN- γ antibody (Figure 4B). Next, we studied STAT1 binding to the Bcl2a1 promoter region in GM-CSF stimulated splenic CD11b⁺ cells from CT-26 tumor bearing mice incubated with and without IFN- γ . Our results show that phosphorylated STAT1 binds to the Bcl2a1 promoter region, indicating a regulatory role of phospho-STAT1 on Bcl2a1 in this system (Figure 6B).

Bcl2a1 overexpression in G-MDSC enhances their survival and suppressive function

To further confirm the effect of Bcl2a1 over-expression on G-MDSC function, we generated a murine congenic transplantation model. Bone marrow cells from B6-Ly5.2/Cr (CD45.1) mice were transduced with lentiviruses expressing GFP alone or GFP / HA-Bcl2a1 prior to reinfusion into sublethally- irradiated C57Bl/6 (CD45.2) recipient mice. Four weeks after transplant 80% of all leukocytes in peripheral blood from mice transplanted with GFP / HA-Bcl2a1 were GFP positive (data not shown). Bcl2a1 transplanted mice were challenged with B16 GM-CSF melanoma and GFP⁺ G-MDSCs were isolated and examined *in vitro*. GFP⁺ G-MDSCs were co-cultured with peptide stimulated OT-I splenocytes and apoptosis was analyzed. As expected, an increase in the frequency of dead (Annexin-V⁺/7-AAD⁺) G-MDSCs was observed after co-culture of G-MDSCs from mice transplanted with a control vector not expressing Bcl2a1 (Figure 7A). This increase was not observed with G-MDSCs from mice transplanted with bone marrow cells transduced with the Bcl2a1 vector. In contrast, Bcl2a1⁺ G-MDSCs showed more suppression of both proliferation (Figure 7B) and IFN- γ production (Figure 7C) by peptide stimulated OT-I TCR T cells than G-MDSCs transduced with the control vector. Finally, we studied the effect of Bcl2a1 overexpression by G-MDSCs *in vivo*. To address this, wild type mice were vaccinated with irradiated B16 GM-CSF cells. Seven days later mice were challenged with B16 tumor cells and granulocytic MDSC isolated either from Bcl2a1 or vector transplanted tumor-bearing littermates were adoptively transferred. Mice developed more tumors after transfer of G-MDSCs isolated from Bcl2a1 transplanted mice than vector control transplanted mice demonstrating a more effective suppressor function *in vivo* (Figure 7D). Our results demonstrate that the over-expression of the anti-apoptotic molecule Bcl2a1 by tumor-induced G-MDSCs leads to a marked functional immunosuppressive phenotype independent of IFN- γ production *in vitro*, which correlates with the suppression of a prophylactic anti-tumor vaccine *in vivo*.

Discussion

MDSCs have recently gained a lot of interest in cancer immunology due to their immune regulatory activity [1, 23]. Two MDSC subtypes have been described in different murine

tumor models, CD11b⁺Gr-1^{dull/int.} G-MDSCs and CD11b⁺Gr-1^{high} G-MDSCs [3, 4], which suppress T-cell responses through different mechanisms. Here, we describe a novel IFN- γ dependent regulatory mechanism of MDSC function. While IFN- γ induces STAT1 phosphorylation in both MDSC subtypes, it has the opposite effect on their suppressor function. It is known that IFN- γ induce NOS2 expression in M-MDSCs with subsequent formation of NO and enhanced suppressor ability [4]. In contrast, G-MDSCs display only minor suppressor activity in the presence of IFN- γ , but become highly suppressive in the absence of IFN- γ . As IFN- γ by itself induced STAT1 phosphorylation in granulocytic MDSCs, it repressed Bcl2a1 expression in these cells and impaired their survival only in the presence of antigen stimulated, GM-CSF expressing T cells and thus resulting in inhibition of their suppressor function.

Our study presents a novel mechanism for granulocytic MDSC function, which is based on their life span after engagement with activated T cells. In general, neutrophils exhibit short life span after attaining effective killing of bacteria or other physiological functions [24]. Here, we demonstrate that Bcl2a1 is up-regulated in tumor-induced G-MDSCs cultured with antigen-stimulated T cells in the absence of IFN- γ . Similar results have been described in naïve and post-effector T cells, in which Bcl2a1 was differentially regulated than Bcl-XL and Bcl-2 [25]. Bcl2a1 is a member of the Bcl-2 family specifically expressed in the hematopoietic cell lineages including macrophages and neutrophils [26]. GM-CSF has been shown to induce Bcl2a1 expression in murine bone marrow cells [27]. A number of studies have demonstrated the role of Bcl2a1 in promoting the longevity of DC and granulocytes [28, 29]. A previous study showed that neutrophils isolated from mice lacking Bcl2a1 undergo accelerated spontaneous apoptosis [30]. *In vitro* IFN- γ was previously shown to down-regulate anti-apoptotic genes such as *BCL2A1* and galectin-3 in melanoma cells [31]. In contrast to our results IFN- γ has recently shown to promote an immunosuppressive phenotype in human neutrophils through PD-L1 expression [32]. However, it should be noted that several studies demonstrated differences between granulocytic MDSCs and normal mouse neutrophils [33, 34]. Since M-MDSCs suppressor activity is mainly dependent on iNOS function [4], we would expect that possible changes in Bcl2a1 expression in M-MDSCs would have less significant effects on their suppressor function. Finally, we also noticed a modest up-regulation of Mcl-1, another Bcl2 family member in G-MDSCs. A similar observation has been made in neutrophils after infection with *Anaplasma phagocytophilum* [35] raising the possibility of an interplay between these two molecules in delaying cell death.

We propose that, while IFN- γ induces STAT1-dependent NOS2 expression in monocytic MDSCs and thereby enhancing their suppressor function, it also impairs survival of granulocytic MDSCs by STAT1 mediated Bcl2a1 repression and thereby inhibiting their suppressor function. In contrast, Bcl2a1 overexpression in granulocytic MDSCs leads to increased survival and enhanced suppression *in vitro*. Most importantly, due to their increased life span G-MDSCs engineered to express Bcl2a1 counteracted vaccination-induced antitumor immunity and promoted tumor occurrence. Stimulation of T cells with their cognate antigen leads to parallel release of GM-CSF and IFN- γ . Here we demonstrate how IFN- γ -induced STAT1 phosphorylation modulates the induction of Bcl2a1 by GM-

CSF in tumor-induced myeloid cells. Therefore, while GM-CSF increases Bcl2a1 expression in a STAT5 dependent manner [36], IFN- γ simultaneously blocks it, providing T cells with an accelerator and brake mechanism to control granulocytic MDSC function in the tumor environment. IFN- γ has been previously shown to display diverse effects on MDSC subsets [4, 15]. Different observations using CD11b⁺Gr-1⁺ cells were also reported [37]. This discrepancy may in part be explained by the cell origin (spleen-derived versus blood-derived). The reason we did not observe any changes in the frequency of G-MDSCs in the spleens of tumor bearing mice both in the presence or absence of IFN- γ could be attributed to the death of granulocytic MDSCs that occur at the tumor environment upon the interaction of activated CTL and is in accordance with published studies [37].

A recent study demonstrated that FasL-Fas mediated killing of MDSCs by T cells is responsible for regulating the homeostasis of MDSC levels *in vivo* [38]. However, it is unclear whether this pathway also plays a role in Bcl2a1-mediated regulation of granulocytic MDSC turnover. High expression levels of anti-apoptotic Bcl-2 family members are associated with resistance of many tumors to chemotherapy, which has led to the development of anticancer drugs that target Bcl-2 [39, 40]. Our study suggests that Bcl2a1 affects the viability of granulocytic MDSCs, which comprise the majority of the suppressive MDSC population. Further studies are required to evaluate whether small Bcl-2 inhibitors interfere with the function of granulocytic MDSCs and eradicate these suppressive cells.

Finally, our results suggest that the heterogeneity of MDSCs allows these cells to suppress immune responses both in the presence or absence of IFN- γ . While monocytic MDSCs become more functional in the presence of IFN- γ , granulocytic MDSCs become more suppressive in the absence of IFN- γ putting the tumor in a “win-win” situation. Furthermore, these findings clearly provide the rationale for future studies to evaluate MDSC survival in more detail to identify new pharmacological targets for inhibiting their suppressor function in the context of tumors.

Materials and methods

Animal models and cell lines

Female six to eight weeks-old wild type BALB/c, B6-Ly5.2/Cr, C57BL/6 and 129Sv mice were purchased from NCI/Frederick. Clone 4 TCR transgenic mice (CL-4) and IFN- γ ^{-/-} were maintained on BALB/c background whereas OT-I TCR and IFN- γ R^{-/-} were bred on C57BL/6 background at NCI/Frederick and Hannover Medical School animal facilities. 129Sv strain STAT1^{-/-} mice were a gift from Christian Capitini (NCI). BALB/c strain IFN- γ ^{-/-} mice were a gift from Siegfried Weiss (Helmholtz Centre for Infection Research, Braunschweig, Germany). B16, B16 GM-CSF, CT-26, EL-4 and RAW264.7 cell lines have been previously described [3, 41]. 344SQ lung cancer cell line was obtained from Jonathan M Curie (MD Anderson Cancer Center, Texas). Mice were injected subcutaneously in the flank with 1×10^6 cells and mice were sacrificed for further analysis when the tumor reached 15 to 20 mm diameter. All mice were handled, fed, and housed in accordance with the U.S. Department of Health and Human Services institutional guidelines.

Flow cytometry

Anti-mouse antibodies against Gr-1, CD11b, Ly6G and Ly6C were purchased from BD Pharmingen. Flow cytometry was performed on a Becton Dickinson FACS Calibur using Cell Quest software (BD). Data analysis was done using FlowJo software (Tree Star). Subsets of MDSCs were purified from spleens of tumor bearing mice by MACS enrichment using anti-mouse CD11b beads (Miltenyi Biotec). MDSC subsets were sorted with anti-Gr-1 and anti-CD11b antibodies (BD Bioscience) using a FACS Aria or Influx cell sorting systems (Becton Dickinson). 7-AAD (BD Pharmingen) was used to exclude dead cells. The purity of MDSC subsets was shown to be more than 95%. Cell viability and cell death were detected with Annexin V-PE (BD Pharmingen) and 7-AAD (BD Pharmingen) staining according to manufacturer's instructions.

Suppression assay and IFN- γ measurement

Different subsets of purified MDSCs (1×10^5 cells) were added to 1×10^5 HA-specific or OVA-specific splenocytes from Clone 4-TCR or OT-I mice respectively at 1:1 ratio in the presence of 0.1 $\mu\text{g/ml}$ HA or OVA-derived SIINFEKL peptide (Biosynthon). After 48 hours IFN- γ concentration in supernatants was measured by ELISA (eBioscience). After 72 hours, ^3H thymidine (Amersham) was added to the cultures for the last 16 hours and proliferation was determined by incorporation of thymidine. Recombinant IFN- γ , GM-CSF, IL-1 β and TNF- α (eBioscience) were added at 100 ng/ml or as mentioned in the figures. 100 μM L-NMMA (Sigma) or nor-NOHA (Sigma) was used when indicated. Anti-IFN- γ (XMG1.2, eBioscience) or anti GM-CSF (MP1-22E9) were added at 100 ng/well to the co-culture experiments.

Microarray analysis

G-MDSCs were FACS sorted after culture with peptide stimulated IFN- $\gamma^{+/+}$ or IFN- $\gamma^{-/-}$ CL-4 T cells. RNA extraction was performed using NucleoSpin RNA II (Macherey-Nagel) followed by Linear T7-based amplification of the RNA. Gene expression analysis was done using PIQOR Immunology Microarray. RNA isolation, amplification and microarray were performed by Miltenyi Biotec. Microarray data was deposited in the GEO database under accession no. GSE32000.

Real Time PCR

Total RNA was isolated from G-MDSCs treated as indicated in the figure using RNeasy kit (Qiagen). cDNA synthesis was carried out using iScript cDNA synthesis kit (Biorad) according to manufacturer's instructions. qPCR (primer sequences available upon request) was performed using IQTM SYBR Green Supermix and thermal cycler (Applied Biosystems). Triplicate reactions were performed for each sample, and expression of tested gene was normalized relative to levels of cyclophilin A.

Intracellular staining for IFN- γ and STAT phosphorylation

Purified M-MDSCs and G-MDSCs from the spleen of CT-26 tumor bearing mice were stained for intracellular IFN- γ according to manufacturer's instructions (BD Pharmingen). For STAT1 and 3 phosphorylation, MDSCs were treated with IFN- γ for 15 minutes and

fixed with 2% paraformaldehyde for 10 minutes at room temperature and permeabilized with 100% ice cold methanol for 30 minutes at 4°C. The cells were washed and stained with anti-phospho STAT1 (pY701) or anti-phospho STAT3 (pY705) antibodies (BD Pharmingen) for 30 minutes.

Bcl2a1 bone marrow-transplanted mice

Bone marrow chimeric mice were generated as previously described [42]. Four weeks after transplantation, 10^6 B16 GM-CSF cells were injected subcutaneously. After 12 days, MDSCs were isolated from splenocytes of the chimera mice. For *in vivo* protection experiments C57BL/6 mice were vaccinated subcutaneously in the left flank with 5×10^5 γ -irradiated B16 GM-CSF followed by adoptive transfer of 1.3×10^6 FACS-sorted G-MDSC on day 7 and subcutaneous challenge with 2×10^5 B16 cells.

ChIP assay

Briefly, 2×10^7 RAW264.7 cells or 1×10^7 splenic CD11b⁺ cells from CT-26 tumor-bearing mice were incubated for 2 hours in the presence of 50 ng/ml GM-CSF. Then, cells were incubated in the presence or absence of 50 ng/ml IFN- γ for 30 minutes. Chromatin immunoprecipitations were performed using anti-phospho-STAT1 antibody (Cell Signaling) and the subsequent controls according to manufacturer's instructions (SimpleChIP Enzymatic Chromatin IP Kit, Cell signaling). Different primer pairs (sequence available upon request) were designed to amplify by real time PCR the 5' region prior to the murine Bcl2a1 promoter. The results obtained were normalized to the 2% input and fold change was calculating considering the background of the negative control.

Statistical analysis

Data were analyzed for statistical significance using Student's t-test. Kaplan-Meier plots were used to analyze survival. Prism software (GraphPad) was used to determine the differences of significance between survival curves with a log-rank test, and differences in other parameters were analyzed using Student's t-test; $p < 0.05$ was considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the Intramural Research Program of the NCI, NIH (ZIA BC 011345) and the by the Initiative and Networking Fund of the Helmholtz Association within the Helmholtz Alliance on Immunotherapy of Cancer.

References

1. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol.* 2009; 9:162–174. [PubMed: 19197294]
2. Peranzoni E, Zilio S, Marigo I, Dolcetti L, Zanovello P, Mandruzzato S, Bronte V. Myeloid-derived suppressor cell heterogeneity and subset definition. *Curr Opin Immunol.* 2010; 22:238–244. [PubMed: 20171075]

3. Haile LA, Gamrekashvili J, Manns MP, Korangy F, Greten TF. CD49d is a new marker for distinct myeloid-derived suppressor cell subpopulations in mice. *J Immunol.* 2010; 185:203–210. [PubMed: 20525890]
4. Movahedi K, Guillemins M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschinn A, De Baetselier P, Van Ginderachter JA. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood.* 2008; 111:4233–4244. [PubMed: 18272812]
5. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol.* 2008; 181:5791–5802. [PubMed: 18832739]
6. Corzo CA, Condamine T, Lu L, Cotter MJ, Youn JI, Cheng P, Cho HI, et al. HIF-1 α regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. *J Exp Med.* 2010; 207:2439–2453. [PubMed: 20876310]
7. Marigo I, Bosio E, Solito S, Mesa C, Fernandez A, Dolcetti L, Ugel S, et al. Tumor-induced tolerance and immune suppression depend on the C/EBP β transcription factor. *Immunity.* 2010; 32:790–802. [PubMed: 20605485]
8. Serafini P, Carbley R, Noonan KA, Tan G, Bronte V, Borrello I. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. *Cancer Res.* 2004; 64:6337–6343. [PubMed: 15342423]
9. Steding CE, Wu ST, Zhang Y, Jeng MH, Elzey BD, Kao C. The role of interleukin-12 on modulating myeloid-derived suppressor cells, increasing overall survival and reducing metastasis. *Immunology.* 2011; 133:221–238. [PubMed: 21453419]
10. Talmadge JE. Pathways mediating the expansion and immunosuppressive activity of myeloid-derived suppressor cells and their relevance to cancer therapy. *Clin Cancer Res.* 2007; 13:5243–5248. [PubMed: 17875751]
11. Sonda N, Chioda M, Zilio S, Simonato F, Bronte V. Transcription factors in myeloid-derived suppressor cell recruitment and function. *Curr Opin Immunol.* 2011; 23:279–285. [PubMed: 21227670]
12. Condamine T, Gabrilovich DI. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. *Trends Immunol.* 2011; 32:19–25. [PubMed: 21067974]
13. Gallina G, Dolcetti L, Serafini P, De Santo C, Marigo I, Colombo MP, Basso G, et al. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8 $^{+}$ T cells. *J Clin Invest.* 2006; 116:2777–2790. [PubMed: 17016559]
14. Ribechini E, Greifengberg V, Sandwick S, Lutz MB. Subsets, expansion and activation of myeloid-derived suppressor cells. *Med Microbiol Immunol.* 2010; 199:273–281. [PubMed: 20376485]
15. Schouppe E, Mommer C, Movahedi K, Laoui D, Morias Y, Gysemans C, Luyckx A, et al. Tumor-induced myeloid-derived suppressor cell subsets exert either inhibitory or stimulatory effects on distinct CD8 $^{+}$ T-cell activation events. *Eur J Immunol.* 2013; 43:2930–2942. [PubMed: 23878002]
16. Mazzoni A, Bronte V, Visintin A, Spitzer JH, Apolloni E, Serafini P, Zanovello P, Segal DM. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J Immunol.* 2002; 168:689–695. [PubMed: 11777962]
17. Bronte V. Myeloid-derived suppressor cells in inflammation: uncovering cell subsets with enhanced immunosuppressive functions. *Eur J Immunol.* 2009; 39:2670–2672. [PubMed: 19757440]
18. Greifengberg V, Ribechini E, Rossner S, Lutz MB. Myeloid-derived suppressor cell activation by combined LPS and IFN- γ treatment impairs DC development. *Eur J Immunol.* 2009; 39:2865–2876. [PubMed: 19637228]
19. Vogler M. BCL2A1: the underdog in the BCL2 family. *Cell Death Differ.* 2012; 19:67–74. [PubMed: 22075983]
20. Sade-Feldman M, Kanterman J, Ish-Shalom E, Elnekave M, Horwitz E, Baniyash M. Tumor necrosis factor- α blocks differentiation and enhances suppressive activity of immature myeloid cells during chronic inflammation. *Immunity.* 2013; 38:541–554. [PubMed: 23477736]

21. Elkabets M, Ribeiro VS, Dinarello CA, Ostrand-Rosenberg S, Di Santo JP, Apte RN, Vosschenrich CA. IL-1 β regulates a novel myeloid-derived suppressor cell subset that impairs NK cell development and function. *Eur J Immunol.* 2010; 40:3347–3357. [PubMed: 21110318]
22. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol.* 2004; 75:163–189. [PubMed: 14525967]
23. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol.* 2009; 182:4499–4506. [PubMed: 19342621]
24. Cross A, Moots RJ, Edwards SW. The dual effects of TNF α on neutrophil apoptosis are mediated via differential effects on expression of Mcl-1 and Bfl-1. *Blood.* 2008; 111:878–884. [PubMed: 17942758]
25. Vershelde C, Walzer T, Galia P, Biemont MC, Quemeneur L, Revillard JP, Marvel J, Bonnefoy-Berard N. A1/Bfl-1 expression is restricted to TCR engagement in T lymphocytes. *Cell Death Differ.* 2003; 10:1059–1067. [PubMed: 12934080]
26. Lin EY, Orlofsky A, Berger MS, Prystowsky MB. Characterization of A1, a novel hemopoietic-specific early-response gene with sequence similarity to bcl-2. *J Immunol.* 1993; 151:1979–1988. [PubMed: 8345191]
27. Orlofsky A, Berger MS, Prystowsky MB. Novel expression pattern of a new member of the MIP-1 family of cytokine-like genes. *Cell Regul.* 1991; 2:403–412. [PubMed: 1832565]
28. Karsan A, Yee E, Harlan JM. Endothelial cell death induced by tumor necrosis factor- α is inhibited by the Bcl-2 family member, A1. *J Biol Chem.* 1996; 271:27201–27204. [PubMed: 8910286]
29. Nopora A, Brocker T. Bcl-2 controls dendritic cell longevity in vivo. *J Immunol.* 2002; 169:3006–3014. [PubMed: 12218115]
30. Hamasaki A, Sendo F, Nakayama K, Ishida N, Negishi I, Nakayama K, Hatakeyama S. Accelerated neutrophil apoptosis in mice lacking A1-a, a subtype of the bcl-2-related A1 gene. *J Exp Med.* 1998; 188:1985–1992. [PubMed: 9841913]
31. Gollob JA, Sciambi CJ, Huang Z, Dressman HK. Gene expression changes and signaling events associated with the direct antimelanoma effect of IFN- γ . *Cancer Res.* 2005; 65:8869–8877. [PubMed: 16204058]
32. de Kleijn S, Langereis JD, Leentjens J, Kox M, Netea MG, Koenderman L, Ferwerda G, et al. IFN- γ -stimulated neutrophils suppress lymphocyte proliferation through expression of PD-L1. *PLoS One.* 2013; 8:e72249. [PubMed: 24015224]
33. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM. Polarization of tumor-associated neutrophil phenotype by TGF- β : "N1" versus "N2" TAN. *Cancer Cell.* 2009; 16:183–194. [PubMed: 19732719]
34. Fridlender ZG, Sun J, Mishalian I, Singhal S, Cheng G, Kapoor V, Horng W, et al. Transcriptomic analysis comparing tumor-associated neutrophils with granulocytic myeloid-derived suppressor cells and normal neutrophils. *PLoS One.* 2012; 7:e31524. [PubMed: 22348096]
35. Pedra JH, Sukumaran B, Carlyon JA, Berliner N, Fikrig E. Modulation of NB4 promyelocytic leukemic cell machinery by *Anaplasma phagocytophilum*. *Genomics.* 2005; 86:365–377. [PubMed: 16005178]
36. Feldman GM, Rosenthal LA, Liu X, Hayes MP, Wynshaw-Boris A, Leonard WJ, Hennighausen L, Finbloom DS. STAT5A-deficient mice demonstrate a defect in granulocyte-macrophage colony-stimulating factor-induced proliferation and gene expression. *Blood.* 1997; 90:1768–1776. [PubMed: 9292509]
37. Sinha P, Parker KH, Horn L, Ostrand-Rosenberg S. Tumor-induced myeloid-derived suppressor cell function is independent of IFN- γ and IL-4 α . *Eur J Immunol.* 2012; 42:2052–2059. [PubMed: 22673957]
38. Sinha P, Chornoguz O, Clements VK, Artemenko KA, Zubarev RA, Ostrand-Rosenberg S. Myeloid-derived suppressor cells express the death receptor Fas and apoptose in response to T cell-expressed FasL. *Blood.* 2011; 117:5381–5390. [PubMed: 21450901]
39. Leber B, Geng F, Kale J, Andrews DW. Drugs targeting Bcl-2 family members as an emerging strategy in cancer. *Expert Rev Mol Med.* 2010; 12:e28. [PubMed: 20822554]

40. Vogler M, Dinsdale D, Dyer MJ, Cohen GM. Bcl-2 inhibitors: small molecules with a big impact on cancer therapy. *Cell Death Differ.* 2009; 16:360–367. [PubMed: 18806758]
41. Sinha B, Eigler A, Baumann KH, Greten TF, Moeller J, Endres S. Nitric oxide downregulates tumour necrosis factor in mRNA in RAW 264.7 cells. *Res Immunol.* 1998; 149:139–150. [PubMed: 9628395]
42. Metais JY, Winkler T, Geyer JT, Calado RT, Aplan PD, Eckhaus MA, Dunbar CE. BCL2A1a over-expression in murine hematopoietic stem and progenitor cells decreases apoptosis and results in hematopoietic transformation. *PLoS One.* 2012; 7:e48267. [PubMed: 23118966]

Figure 1. IFN- γ has a differential effect on the suppressive function of MDSC subsets

(A) Sorted M-MDSCs and G-MDSCs were co-cultured with antigen-specific CL-4 TCR splenocytes at the indicated ratios in the presence of HA peptide, and proliferation was measured. (B and C) Sorted G-MDSCs (B) and M-MDSCs (C) from tumor bearing mice were co-cultured with antigen-specific CL-4 TCR splenocytes at 1:1 ratio in the presence of HA peptide, and proliferation was measured. 100 μ M L-NMMA, nor-NOHA and 100 ng/ml anti-IFN- γ antibody were added simultaneously at the beginning of the culture as indicated in the figure. (A–C) Each experiment was done in triplicate with (A) $n > 3$ or (B, C) $n=3$ mice per group. Data are shown as mean $\pm$ SD are representative of one experiment from (A) two or (B,C) three independent experiments. * $p < 0.05$; Student's t test.

Figure 2. IFN- γ impairs the suppressor function of G-MDSCs

(A) Sorted M-MDSCs and G-MDSCs from tumor bearing mice were co-cultured with antigen-specific IFN- γ ^{-/-} CL-4 TCR splenocytes and exogenous IFN- γ (100 ng/ml) was added at the start of the culture. (B) Sorted G-MDSCs from tumor bearing mice were co-cultured with antigen-specific CL-4 TCR splenocytes with or without the indicated concentration of exogenous IFN- γ . $p < 0.05$ (C) Sorted M-MDSCs from CT-26 tumor bearing mice were co-cultured with antigen-specific CL-4 TCR splenocytes at the indicated concentrations of exogenous IFN- γ . (D) Purified M-MDSCs and G-MDSCs from EL-4 tumor bearing IFN- γ R^{-/-} mice were co-cultured with OVA-specific splenocytes in the presence of 0.1 μ g/ml OVA peptide at 1:1 ratio. (A–D) Data are shown as mean $\pm$ SD. Each experiment was performed in triplicate with $n = 2$ –3 mice per group. Data shown are representative of two independent experiments. * $p < 0.05$; Student's t test.

Figure 3. Anti-apoptotic Bcl2a1 is specifically up regulated on G-MDSC in the absence of IFN- γ
 FACS-sorted splenic G-MDSCs from CT-26 tumor-bearing mice were re-isolated upon co-culture either with WT or IFN- $\gamma^{-/-}$ CL-4 TCR splenocytes and mRNA expression of Bcl2a1 was measured by quantitative PCR after (A) 12 and (B) 48 hours. Expression was set relative to cyclophilin A mRNA. (C) FACS-sorted splenic G-MDSCs from CT-26 tumor-bearing mice were co-cultured with CL-4 TCR splenocytes and IFN- γ blocking antibody was added when indicated. Cells were cultured for 48 hours, then G-MDSCs were resorted. Bcl2a1 expression was set relative to cyclophilin A mRNA. (D and E) Sorted splenic G-

MDSCs were cultured either with WT or IFN- $\gamma^{-/-}$ CL-4 splenocytes (1:1 ratio) in the presence of HA peptide. Cells were cultured for 48 hours, then G-MDSCs were resorted. Anti-apoptotic (black bar) and pro-apoptotic (white bar) gene expression was set relative to cyclophilin A mRNA. (A–E) Data are shown as mean +SD. Each experiment was performed in triplicate with (A, B) n=4 or (C–E) n=3 mice in each experiment. Results shown are representative of two independent experiments. *p<0.05; Student's t test.

Figure 4. GM-CSF induces Bcl2a1 in G-MDSCs

(A) Purified splenic G-MDSCs from CT-26 tumor-bearing mice were cultured with recombinant GM-CSF at the indicated concentrations for 24 hours. Quantitative PCR was performed for the expression of Bcl2a1 mRNA. (B) Bcl2a1 mRNA expression was quantified using qPCR on G-MDSCs cultured alone with IFN- $\gamma^{-/-}$ CL-4 TCR in the presence and absence of exogenous IFN- γ . (C and D) Purified G-MDSCs were cultured either with IFN- $\gamma^{+/+}$ or IFN- $\gamma^{-/-}$ CL-4 TCR antigen-stimulated splenocytes in the absence or presence of IFN- γ as indicated. The frequency of Annexin-V/7-AAD negative cells was determined via FACS. (C) Representative dot plots for Annexin-V and 7-AAD gated on CD11b⁺Gr-1^{high} cells as well as (D) quantification are shown. (A–D) Data are shown as mean +SD of n=3

mice in each experiment and are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$; Student's *t* test..

Figure 5. IFN- γ inducible genes are not altered in G-MDSCs and STAT1 controls the expression of Bcl2a1

(A) Sorted M-MDSCs and G-MDSCs from CT-26 tumor bearing mice were treated with IFN- γ (100 U/ml) for 15 minutes. Intracellular staining for phosphorylated STAT1 and STAT3 was performed as described in the *Materials and methods*. Upper histograms show staining for CD11b⁺Gr-1^{dull/int.} cells and lower histogram depict CD11b⁺Gr-1^{high} cells. Filled histograms show without stimulation with IFN- γ and open histograms indicate after stimulation with IFN- γ . Data shown is representative from two independent experiments. (B) 344SQ cells were injected in 129S1/SvImJ/Cr and STAT1^{-/-} mice. The frequency of MDSC subsets in the spleen was quantified by flow cytometry when the tumor reached 15 mm. (C) M-MDSC and G-MDSC subsets were purified from the spleen of 344SQ tumor bearing 129S1/SvImJ/Cr and STAT1^{-/-} mice. MDSC subsets were cultured with antigen specific OT-I splenocytes in the presence of OVA peptide (0.1 μ g/ml) for 72h. Proliferation was measured by thymidine incorporation. (D) Purified WT and STAT1^{-/-} G-MDSCs were cultured alone or together with OT-I TCR splenocytes in the presence of OVA peptide. After 12 hours, G-MDSCs were re-sorted and the expression of Bcl2a1 was quantified by

qPCR. Expression was set relative to cyclophilin A mRNA. (B–D) Data are shown as mean +SD of n=3 mice and are representative of two independent experiments, each one performed in triplicates. Student's t test. *p<0.05.

Figure 6. IFN- γ mediated activation of Stat1 represses Bcl2a1 expression on myeloid cells
 (A) RAW264.7 cells were incubated for 2 hours with 50 ng/ml GM-CSF and 50 ng/ml IFN- γ were added for 30 minutes as indicated. Cells were harvested and Bcl2a1 mRNA expression levels were determined by qPCR. Bcl2a1 gene expression was set relative to cyclophilin A mRNA. (B) Splenic CD11b⁺ cells from CT-26 tumor-bearing mice were stimulated with GM-CSF and cultured with and without IFN- γ . Samples were subjected to pSTAT1 chromatin immunoprecipitation (ChIP). Quantitative analysis of precipitated DNA or 2% of chromatin input with gene-specific primers amplifying Bcl2a1 proximal promoter regions were performed. (A, B) Data are shown as mean $\pm$ SD of n=3 samples and are (A) representative of two independent experiments or (B) cumulative of two independent experiments and normalized to the IgG background signal. *p<0.05, **p<0.01; Student's t test.

Figure 7. Bcl2a1 overexpression by CD11b⁺Gr-1^{high} MDSC enhances their immunosuppressive abilities in vitro and in vivo

(A) Chimeric mice generated by transplanting Lin^{neg} BM cells transduced with either a Bcl2a1 or with a control vector were inoculated s.c. with B16 GM-CSF tumor cells. After sacrifice 10⁵ sorted splenic GFP⁺ G-MDSCs were cultured alone or with 10⁵ OT-I splenocytes in the presence of 0.1 μg/ml OVA. Anti-IFN-γ (10 μg/ml) was added to the co-culture when indicated. The frequency of Annexin-V/7-AAD positive cells was determined by FACS on the second day. (B, C) GFP⁺ G-MDSC cells were sorted from Bcl2a1 or control group mice bearing B16 GM-CSF tumors. 10⁵ sorted cells were cultured alone or with 10⁵ OT-I splenocytes in the presence of 0.1 μg/ml OVA peptide. Proliferation (B) and IFN-γ production (C) in the co-culture were measured by ³H assay and ELISA respectively. (D) C57 BL/6 mice were vaccinated with γ-irradiated B16 GM-CSF cells on day 0. On day 7, 1.3×10⁶ G-MDSCs from B16 GM-CSF tumor-bearing mice from either Bcl2a1 or vector BM chimeras were adoptively transferred and mice were challenged with 2×10⁵ B16 cells. Kaplan-Meier plot of tumor-free mice is shown. (A, D) Data are shown as mean +SD of (A–C) n=4 or (D) n=10 mice per group and are (A) representative of one out of two independent experiments or (B–D) pooled from two independent experiments.. *p < 0.05; **p < 0.005; (A–C) Student's t test, (D) log-rank test.

Table 1

List of genes differentially expressed genes in CD11b⁺Gr-1^{high} cells upon incubation with antigen-stimulated IFN- γ ^{-/-} T cells (complete data is deposited in the GEO database under accession no. GSE32000).

Gene		Fold change
<i>Bcl2a1</i>	BCL2-related protein A1	52.630
<i>Socs1</i>	suppressor of cytokine signaling 1	14.800
<i>Cd14</i>	CD14 molecule	13.220
<i>Cish</i>	Cytokine inducible SH2-containing protein	11.830
<i>Tlr4</i>	Toll-like receptor 4	3.070
<i>Cebpβ</i>	CCAAT/enhancer binding protein (C/EBP), beta	2.780
<i>Bnip3</i>	BCL2/adenovirus E1B 19kDa interacting protein 3	2.500
<i>Bcl2l1</i>	BCL2-like 1	2.360
<i>Bcl2l11</i>	BCL2-like 11	1.700
<i>Mcl1</i>	Myeloid cell leukemia sequence 1 (BCL2-related)	1.360