Molecular control of myeloid suppressors by death pathways

Jessica M. Haverkamp et al.

Supplemental Material

Index

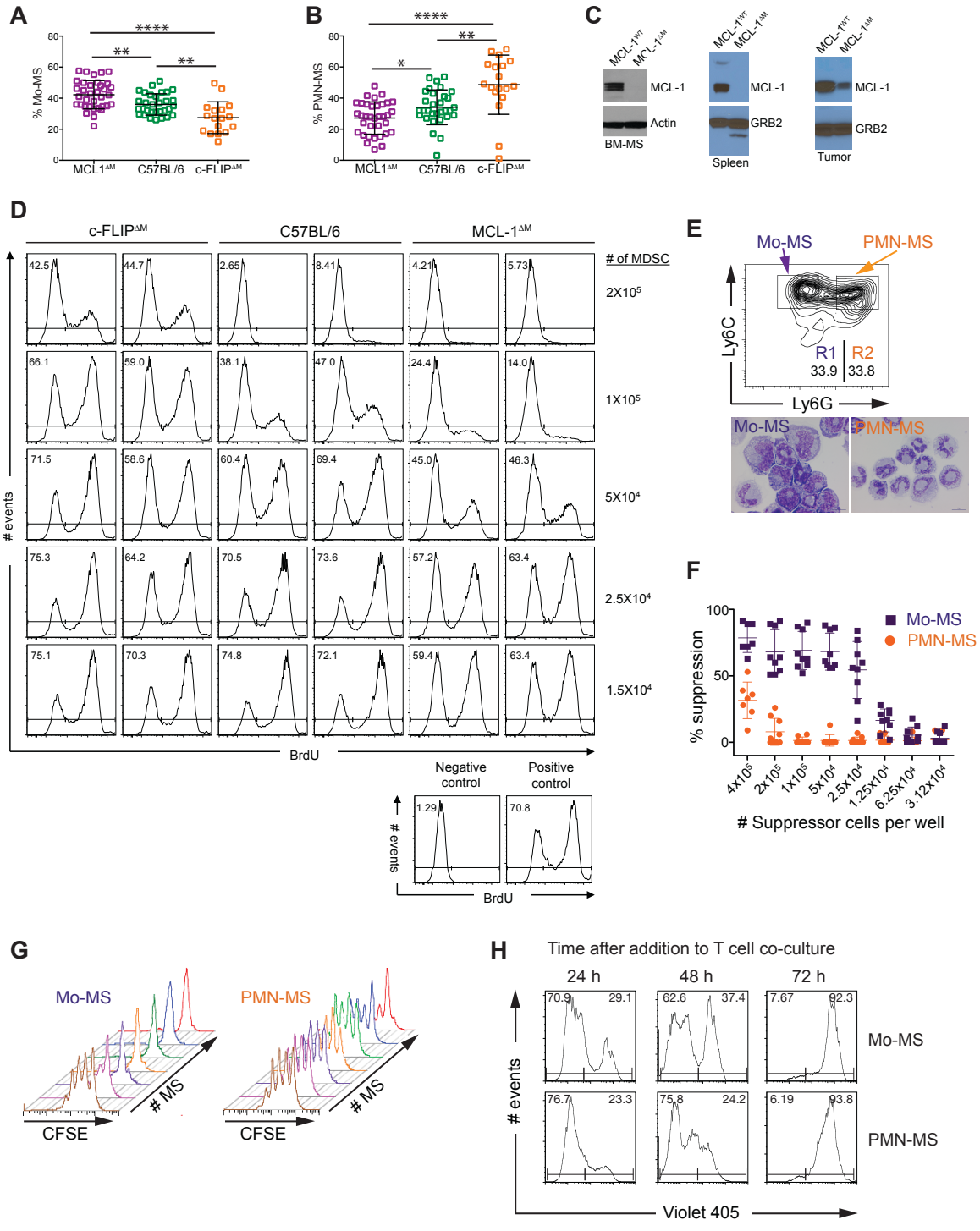

Figure S1, related to Figure 1.

Mo-MS are the dominant immunosuppressive MS population.

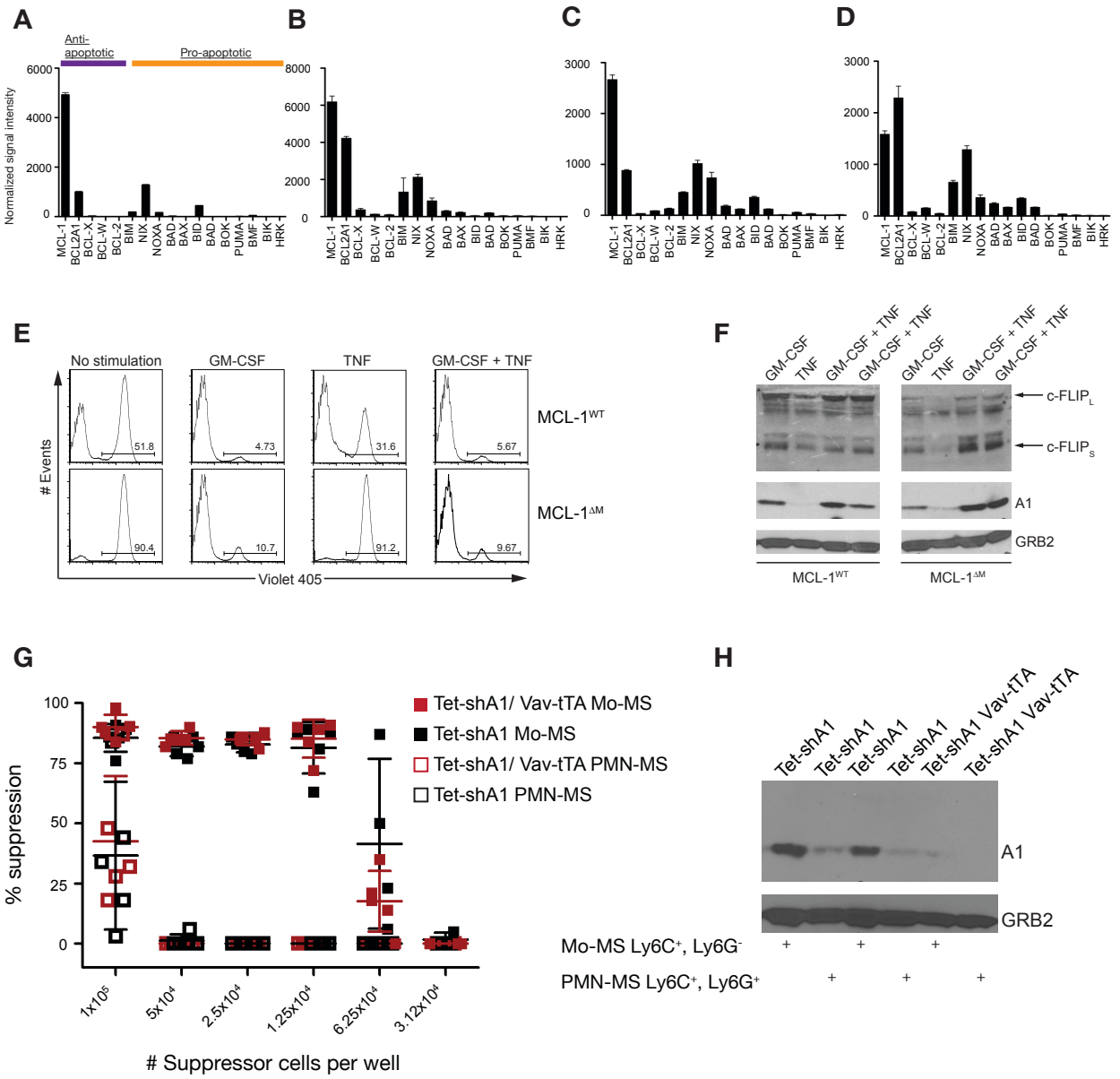

Figure S2, related to Figure 3.

The apoptotic regulators MCL-1 and A1 are highly expressed in human and mouse myeloid suppressor cells.

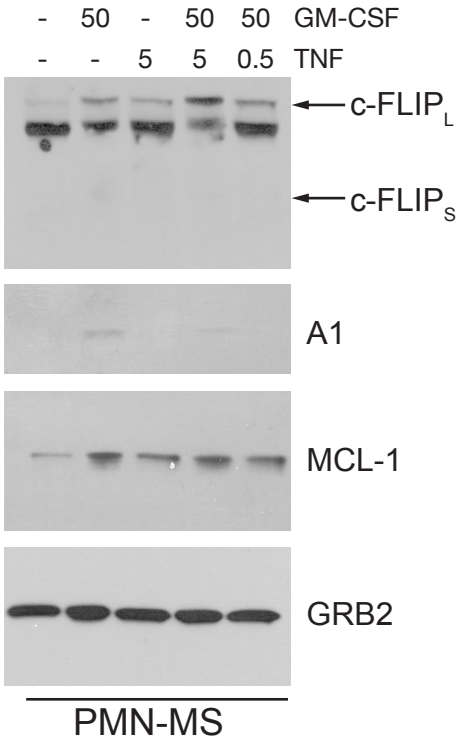

Figure S3, related to Figure 4.

Expression of anti-apoptotic regulators in PMN-MS.

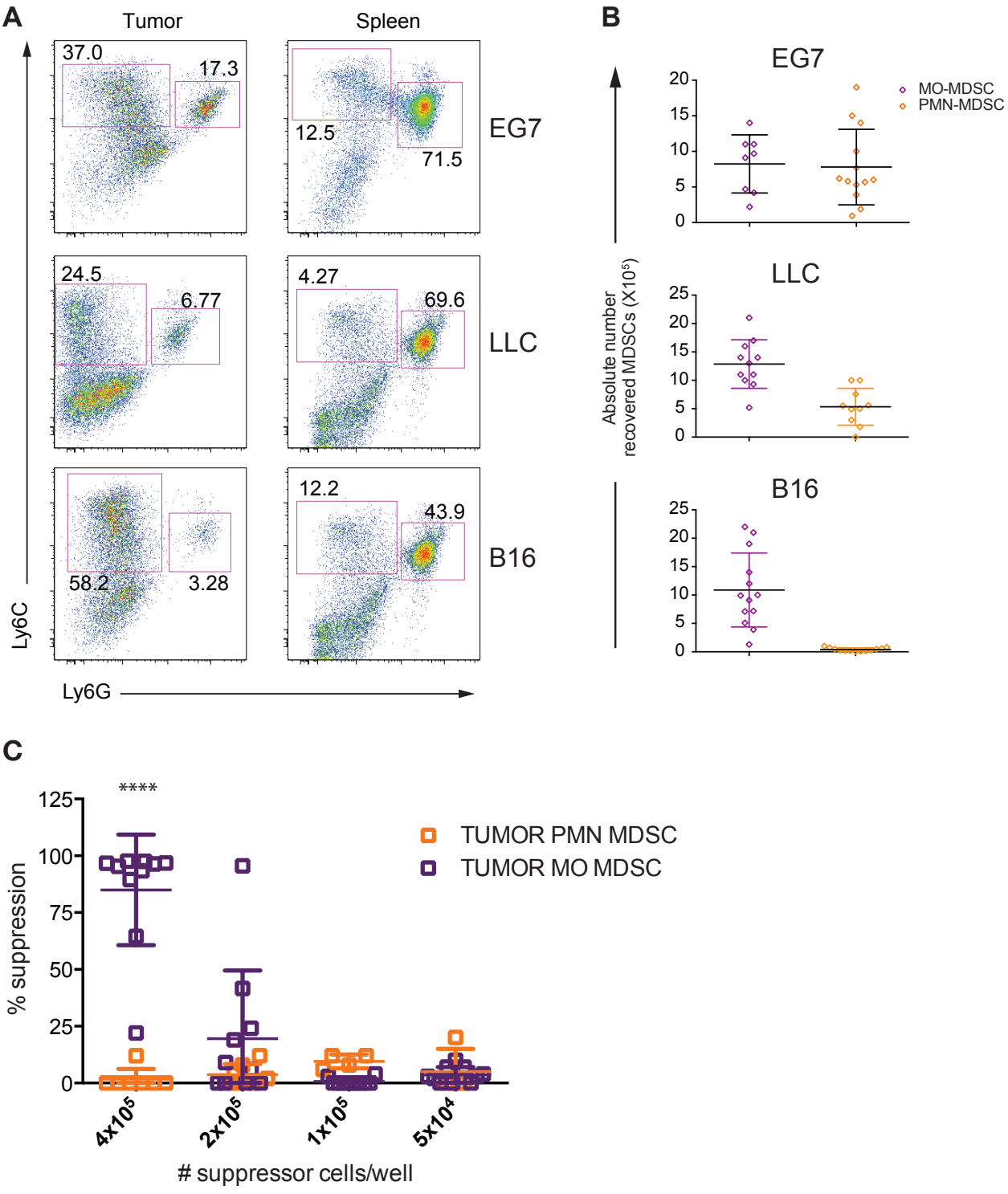

Figure S4, related to Figure 5.

Phenotypic and functional analysis of Mo and PMN-MDSCs in cancer

Supplemental Figure Legends

Figure S1, related to Figure 1.

Fidelity of the MCL-1$^{\Delta M}$ and c-FLIP$^{\Delta M}$ conditional KO mouse models.

(A-B) The percentage of Mo-MS (A) and PMN-MS (B) in CD11b^{+} gated BM-MS cultures from MCL-1$^{\Delta M}$, C57BL/6 and c-FLIP$^{\Delta M}$ mice. Data are pooled from 20 independent experiments (n=17-35 per group). Mice were analyzed individually. Graphs show mean ± s.d.*p<0.01, **p<0.001, ****<0.0001.

(C) MCL-1 protein is efficiently deleted in MCL-1$^{\Delta M}$ mice. Protein lysates were made from BM-MS and from spleen and tumor resident Mo-MDSC isolated from EG7 tumor bearing MCL-1^{WT} and MCL-1$^{\Delta M}$ mice. Samples were subjected to immunoblotting for MCL-1 and actin or GRB2 as a loading control. Residual MCL-1 protein in the MCL-1$^{\Delta M}$ tumor samples may come from cells selected for non-deletion with the LysM-Cre (Vannella et al., 2014), or from MCL-1^{+} tumor cells phagocytosed by tumor resident CD11b^{+} Mo-MDSCs, as MCL-1 is highly expressed in cancerous cells (Belmar and Fesik, 2014).

(D) BM-MS were generated from c-FLIP$^{\Delta M}$, C57BL/6, and MCL-1$^{\Delta M}$ mice and suppressive function was measured by monitoring BrdU incorporation in OT-I cells wherein 5x10^{5} OT-I cells were co-cultured for 72 h with titrated MS in the presence of SIINFEKL peptide. As a negative control OT-I cells were cultured in RMPI only and as a positive control OT-I cells were cultured with SIINFEKL peptide in the absence of MS cells. Values represent the percentage of BrdU^{+} cells among the total Thy1.2^{+} CD8^{+} OT-I cells. Data are representative of 3 independent experiments (n = 2 mice for each experiment).

(E) Representative gating strategy to separate monocytic-MS (Ly6G^{-}, Ly6C^{+}; R1, Mo-MS) and granulocytic-MS (Ly6G^{+}, Ly6C^{+}; R2, PMN-MS) from C57BL/6 mice. Below panel E are representative cytospins showing the morphology of the sorted MDSC populations.

(F, G) Suppressive function was measured using 5 x 10^{5} CFSE-labeled OT-I cells co-cultured with titrated MS in the presence of SIINFEKL peptide. CFSE dilution was evaluated by flow cytometry after 72 h. Data in panel F is complied from multiple independent experiments (n = 7-8). (G) Representative cascade plots from 3 independent experiments, showing CFSE dilution in

OT-I cells cultured with decreasing numbers of MS. Plots were gated on $CD8^+$ cells. Percent suppression of proliferation was calculated as described in Materials and Methods. Data are compiled from 3 independent experiments (n = 7), and are presented as the mean ± s.d.
(H) BM-MS were generated from C57BL/6 mice as described in Materials and Methods. Mo- and PMN-MS were sorted and cultured at 2 x 10^5 cells per well with 5 x 10^5 OT-I cells in the presence of SIINFEKL peptide. The percentage of viable MS in T cell suppression assay cultures was evaluated at 24 h intervals during the assay using V405 staining of $Ly6C^+$ cells at the indicated time points.

Figure S2, related to Figure 3.

The apoptotic regulators MCL-1 and A1 are highly expressed in human and mouse myeloid suppressor cells.

(A-D) Signal intensity of mRNAs encoding apoptotic regulators expressed in (A) human $CD14^+$, $HLA\text{-}DR^{lo}$ MDSCs isolated from blood of head and neck cancer patients (n = 3), (B) bulk $CD11b^+$ cells isolated from C57BL/6 mice bearing EG7 tumors (n = 3) (C) $Ly6G^{hi}$ depleted, $Ly6C^+$ cells from spleens of C57BL/6 mice bearing EG7 tumors (n = 2), (D) in vitro generated murine BM-MS (n = 7). Data are the mean signal from affymetrix microarrays ± SEM.
(E) Viability of Mo-MS from MCL-$1^{\Delta M}$ or control MCL-1^{WT} mice was examined after 24hr culture in the presence of the indicated cytokines: GM-CSF (50 ng/ml), TNF (5 ng/mL).
(F) BM-MS from MCL-$1^{\Delta M}$ or control MCL-1^{WT} mice and Mo-MS were sorted as described in materials and methods. Mo-MS were cultured for 24 hr in the presence of the indicated cytokines: GM-CSF (50 ng/ml), TNF (5 ng/mL); GM-CSF (50 ng/ml) + TNF (5 ng/mL); GM-CSF (50 ng/mL) + TNF (0.5 ng/mL). Cell lysates were subjected to immunoblotting for the indicated proteins.
(G) BM-MS were generated from Tet-ShA1/Vav-tTA (A1 knockdown) or Tet-ShA1 (control) mice and suppressive function was monitored by CFSE dilution. 5x10^5 OT-I cells were cultured with MDSCs grown from individual mice (n = 5) for 72 hrs. Percent suppression of proliferation was calculated as described in Materials and Methods. Data are combined from 2 independent experiments, and are presented as the mean ± s.d.

(H) Verification of A1 knockdown by immunoblotting in Tet-ShA1/Vav-tTA PMN and Mo-MS relative to Tet-ShA1 control cells.

Figure S3, related to Figure 4.

Expression of anti-apoptotic regulators in PMN-MS.

(A) BM-MS were grown from C57BL/6 mice. PMN-MS were stimulated for 24 h with the indicated cytokines and cell lysates were subjected to immunoblotting for the indicated targets. GM-CSF (50ng/mL), TNF (5 ng/mL and 0.5 ng/mL). GRB2 (~ 26 kDa) was used as the loading control.

Figure S4, related to Figure 5.

Phenotypic and functional analysis of Mo and PMN-MDSCs in cancer

(A-B) Phenotypic analysis of spleen and tumor resident Mo- and PMN-MDSCs from C57BL/6 mice bearing flank EG7, LLC, or B16 tumors. $CD11b^+$ cells were isolated by MACS and subsequently evaluated by flow cytometry. (A) Contour plots show the percentage of Mo- ($Ly6G^-Ly6C^+$) and PMN-MDSC ($Ly6G^+Ly6C^+$) and are gated on live cells following $CD11b^+$ MACS isolation. Data are representative of 2 independent experiments (n= 5-10 mice per group,). (B) The absolute number of Mo- and PMN-MDSC recovered from tumor tissue. Data are generated from contour pots gated as in (A). Data are compiled from 2 independent experiments (n= 5-10 mice per group, per experiment).

(C) LLC tumors were grown on the flank of C57BL/6 mice and after 14 days tumor resident Mo and PMN-MDSC were evaluated for immunosuppressive function as described in Materials and Methods. Percent suppression is calculated as described in the methods. Statistical analysis with unpaired t tests was performed; ****$p < 0.0001$. Data are expressed as the mean $\pm$ s.d. and are compiled from 2 independent experiments (n= 5-10 mice per group, per experiment).

Supplemental experimental procedures

Generation of mouse BM-MS

MDSCs were generated from the bone marrow (BM) (Marigo et al., 2010). BM was isolated and cleared of red blood cells with red blood cell lysis buffer. Following centrifugation, 2.5 x 10^6 cells were plated in 10 ml RPMI containing 10% FBS, antibiotics, 40 ng/ml GM-CSF and 40 ng/ml IL-6 (both from Becton Dickinson) for 6 days. The non-adherent fraction was removed and the adherent fraction treated with PBS + 1 mM EDTA to dislodge the cells (Marigo et al., 2010). Adherent and non-adherent fractions were pooled and counted. Quality control for each preparation was performed by cytospin and Ly6G/Ly6C staining. Where indicated inducible deletion of *Cflar* was achieved by administration of 4-OH-tamoxifen at various time points during BM culture. For these studies control cultures were given vehicle control (ethanol). Where indicated MS cultures were treated with QVD-OPH (SM Biochemicals).

Isolation of leukocytes from spleens and lymph nodes

Leukocytes were isolated from spleens and lymph nodes of mice by pressing tissue against a 70 μm filter. Red blood cells in spleen cell suspensions were lysed with red blood cell lysis buffer.

Immunoblotting

Procedures for immunoblots with myeloid cells were previously described (El Kasmi et al., 2008). Antibodies used in this study include primary anti-MCL-1 (Rockland), anti-A1 mAb (from M. Herold), and anti-GRB2 (BD Pharmingen). Antibodies to c-FLIP were purchased from Cell Signaling (Rabbit mAb D16A8, order number #8510) and used in accordance with their guidelines and published information on the specificity of anti-c-FLIP antibodies (Bucur et al., 2013), and through the use of c-FLIP-deficient MS lysates were used as a specificity control.

Isolation of Human MDSCs from cancer patients

Human MDSCs characterized as $CD14^+$ $HLA\text{-}DR^{-/low}$ were isolated from freshly obtained peripheral blood with consent from Head & Neck Squamous Cell Carcinoma patients undergoing

surgical treatments at Johns Hopkins Hospital and Johns Hopkins Bayview Hospital. $CD11b^{+}$ $CD14^{+}$ $HLA\text{-}DR^{-/low}$ cells were sorted on a MoFlo MLS sorter (Beckman Coulter) or a FACSAria II cell sorter (Becton Dickinson) at Johns Hopkins Hospital Cell Cytometry Core Facility after a Ficoll purification step. Anti-human anti-CD14, anti-CD11b, anti-HLA-DR (eBioscience, San Diego CA) were used for sorting.

Gene expression analysis by microarray

RNA quality was confirmed by analysis on the Agilient 2100 Bioanalyzer. Biotin-labeled targets were generated from 100 ng total mouse RNA using the Affymetrix 3'IVT Express assay or from 20 ng total human RNA using the NuGEN WT-Ovation Pico assay as per the manufacturers' recommended protocols. Five µg of labeled targets were hybridized overnight to the HT HG-U133+ PM array (human samples) or to the HT MG-430 PM array (mouse samples), then processed using the Affymetrix GeneTitan system. Signals from scanned arrays were summarized to transcript (probe set) measures using the RMA algorithm (Affymetrix Expression Console v1.1) and then converted to linear signals by exponentiation (base 2). Probe set annotations were obtained from the Affymetrix NetAffx website. Gene expression values were calculated as the maximum signal across redundant probe sets and are reported as the mean signal +/- SEM.

References

Belmar, J., and Fesik, S.W. (2014). Small molecule Mcl-1 inhibitors for the treatment of cancer. Pharmacol Ther.

Bucur, O., Pennarun, B., Stancu, A.L., Nadler, M., Muraru, M.S., Bertomeu, T., and Khosravi-Far, R. (2013). Poor antibody validation is a challenge in biomedical research: a case study for detection of c-FLIP. Apoptosis *18*, 1154-1162.

El Kasmi, K.C., Qualls, J.E., Pesce, J.T., Smith, A.M., Thompson, R.W., Henao-Tamayo, M., Basaraba, R.J., Konig, T., Schleicher, U., Koo, M.S.*, et al.* (2008). Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. Nat Immunol *9*, 1399-1406.

Marigo, I., Bosio, E., Solito, S., Mesa, C., Fernandez, A., Dolcetti, L., Ugel, S., Sonda, N., Bicciato, S., Falisi, E*., et al.* (2010). Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor. Immunity *32*, 790-802.

Vannella, K.M., Barron, L., Borthwick, L.A., Kindrachuk, K.N., Narasimhan, P.B., Hart, K.M., Thompson, R.W., White, S., Cheever, A.W., Ramalingam, T.R*., et al.* (2014). Incomplete Deletion of IL-4Ralpha by LysMCre Reveals Distinct Subsets of M2 Macrophages Controlling Inflammation and Fibrosis in Chronic Schistosomiasis. PLoS Pathog *10*, e1004372.