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Myeloid-derived suppressor cell (MDSC) immunomodulation by c-FLIP

MED/04

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ABBREVIATION LIST

APC: Antigen Presenting Cells

ARG1: Arginase 1

ATP: Adenosine TriPhosphate

BM: Bone Marrow

BM-MDSC: Bone Marrow-derived Myeloid-Derived Suppressor Cells

CC(R/L)2: C-C chemokine (Receptor/Ligand) type 2

CD: Cluster of Differentiation

CDK4:Cyclin-Dependent Kinase 4

CDKN2A: Cyclin-Dependent Kinase iNhibitor 2A

CDP: Common DC precursor

c-FLIP: (FADD-like IL-1β-converting enzyme)-inhibitory protein

CSF-1: Colony Stimulating Factor 1

COX-2: CycloOXygenase-2

CTL: Cytotoxic T Lymphocyte

CXCL/R: chemokine (C-X-C motif) Ligand/Receptor

DC: Dendritic Cell

ECM: Extra-Cellular Matrix

EMT: Epithelial to Mesenchymal Transition

FBS: Fetal Bovine Serum

Fc: Fragment crystallizable

Flt3L: FMS-Like Tyrosine kinase 3 Ligand

FMO: Fluorescence-Minus-One

G-CSF: Granulocyte Colony Stimulating Factor

GM-CSF: Granuloyte-Macrophage Colony Stimulating Factor

GvHD: Graft-Versus-Host Disease

HD: Healthy Donor

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HGF: Hepatocyte Growth Factor

HIF1α: Hypoxia Inducible Factor-1α

HLA: Human Leukocyte Antigen

HSC: Hematopoietic Stem Cell

iDC: Immature DC

IDO: Indoleamine-pyrrole 2,3-DiOxygenase

IFN-γ: InterFeroN Gamma

Ig: ImmunoGlobulin

IL: InterLeukin

IL-1RA: InterLeukin-1 Receptor Antagonist

IL-2Rγ: gamma subunit of the InterLeukin 2 Receptor

IL4Ra: alpha subunit of the InterLeukin 4 Receptor

IMC: Immature Myeloid Cell precursor

IMDM: Iscove's Modified Dulbecco's Medium

IPMN: Intraductal Papillary Mucinous Neoplasm

KIR: Killer Ig-like Receptor

KRAS: v-Ki-ras2 Kirsten RAt Sarcoma viral oncogene homolog

LDL: Low-Density Lipoprotein

LIN: LINeage

LP: Lymphoid multipotent Precursor

MCL1: Induced myeloid leukemia cell differentiation

M-CSF: Macrophage Colony Stimulating Factor

MCN: Mucinous CystadeNomas

MCP-1: Monocyte Chemoattractant Protein 1

MDSC: Myeloid-Derived Suppressor Cells

MHC: Major Histocompatibility Complex

MMP: Matrix MetalloProteinase

MNC: MonoNuclear Cell

MOI: Multiplicity Of Infection

M-MDSC: Monocytic Myeloid-Derived Suppressor Cell

MP: Myeloid multipotent Precursor

MRC1: Mannose ReCeptor 1

NF-kB: Nuclear Factor-kappa B

NK: Natural Killer

NN: Naïve Neutrophil

NO: Nitric Oxide

NOG: NODShi.Cg-PrkdcscidIl2rG

NOS2: Nitric-Oxide Synthase type 2

NOX: NADPH OXidase

NS: Natural Suppressor cell

NSG: NOD.Cg-PrkdcScidIl2rG

PBMC: Peripheral Blood Mononuclear Cell

PBS: Phosphate Buffered Saline

pDC: plasmacytoid Dendritic Cell

PDE5: PhosphoDiEsterase-5

PDGF: Platelet Derived Growth Factor

PDL-1: Programmed Death Ligand-1

PDX: Patient Derived Xenograft

PMN-MDSC PolyMorphoNuclear Myeloid-Derived Suppressor Cell

Rb1: RetinoBlastoma protein

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RPMI: Roswell Park Memorial Institute

SCF: Stem Cell Factor

SD: Standard Deviation

ShRNA: Short Hairpin RNA

SIRPα: SIgnal Regulatory Protein α

SMAD4: Mothers Against Decapentaplegic homolog 4

SOCS2: Suppressor Of Cytokine Signaling 2

TAM: Tumor-Associated Macrophage

TAN: Tumor-Associated Neutrophil

TBI: Total Body Irradiation

TCR: T Cell Receptor

TDF: Tumor-Derived Factor

TGF-α: Transforming Growth Factor alpha

TGF-β: Transforming Growth Factor beta

TIC: Tumor-Initiating Cell

Tip-DC: TNF and Inducibile iNOS-Producing DC

TNF: Tumor Necrosis Factor

TP53: Tumor Protein p53

TPO: ThromboPOietin

Treg: Regulatory T cell

VEGF: Vascular Endothelial Growth Fact

SOMMARIO

Durante il processo tumorigenico, le cellule tumorali secernono diversi fattori, quali citochine, chemochine e altri metaboliti, in grado di promuovere lo svilupparsi di un microambiente plastico caratterizzato da una continua angiogenesi e da importanti modifiche del sistema immunitario (Balkwill, Charles et al. 2005). Una delle strategie più efficaci attuata dal tumore per eludere il sistema immunitario è la capacità di creare un microambiente tollerogenico attraverso il rimodellamento del normale processo di ematopoiesi. Infatti, il tumore può indurre la proliferazione ed il differenziamento di precursori mieloidi in cellule mature con marcate proprietà immunosoppressive. Queste cellule, chiamate cellule soppressorie di derivazione mieloide (MDSCs), costituiscono una popolazione cellulare eterogenea che si espande in alcune condizioni patologiche, come le neoplasie, le infiammazioni e le infezioni ed hanno la capacità di inibire potentemente l'immunità antitumorale mediata dai linfociti T. La presenza e espansione di queste cellule limita notevolmente lo sviluppo di nuovi protocolli di immunoterapia anti-tumorale. In questo studio abbiamo dimostrato la proprietà di basse dosi di diversi chemioterapici, caratterizzati da differenti bersagli molecolari e da diversa attività citotossica, largamente impiegati nella pratica clinica, di depletare in modo selettivo le MDSC monocitarie (M-MDSC), ricostituendo completamente il potenziale proliferativo dei linfociti T. Inoltre, abbiamo dimostrato che le varie formulazioni farmacologiche effettuano la loro azione citotossica sulle MDSC attivando la via estrinseca dell'apoptosi e, in particolare, modulando negativamente l'espressione della proteina cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP), la cui attività anti-apoptotica è stata largamente descritta in letteratura. In particolare, un recente studio ha dimostrato come l'eterogeneità delle MDSC nelle due sue sottopopolazioni, monocitarie (M-MDSC) e polimorfonucleate (PMN-MDSC), risiede nella diversa attivazione delle vie dell'apoptosi: le PMN-MDSC necessitano della proteina anti-apoptotica MCL-1 per la loro corretta maturazione, mentre il differenziamento e la sopravvivenza

delle M-MDSC necessitano di c-FLIP (Haverkamp, Smith et al. 2014). Pertanto, abbiamo verificato l'ipotesi che c-FLIP potesse avere anche un ruolo chiave nell'indurre e controllare l'attività immunosoppressiva delle MDSC. Abbiamo, quindi, dimostrato che l'aumentata espressione di c-FLIP in cellule di origine monocitaria, attraverso l'infezione con costrutti lentivirali, è sufficiente per indurre un programma soppressivo dominante, caratterizzato dall'up-regolazione di svariate proteine e citochine già note in letteratura per essere in grado di espletare l'attività soppressiva delle MDSC. L'aumentata espressione di c-FLIP può quindi generare potenti cellule mieloidi soppressorie, in grado di inibire la proliferazione e attivazione di linfociti T, sia *in vitro* che *in vivo* in un modello di graft versus host disease (GvHD) xenogenico dimostrando come tale strategia immunoterapica possa avere un elevato potenziale di traslabilità terapeutica. In aggiunta, abbiamo dimostrato la capacità di c-FLIP di promuovere proprietà immunomodulatorie anche utilizzando monociti derivati da un modello murino transgenico caratterizzato dall'over-espressione dell'isoforma virale di c-FLIP, vFLIP, nelle cellule mieloidi. Anche in questo caso le cellule mieloidi hanno presentato un'estrema capacità soppressoria sia *in vitro* che *in vivo*.

L'up-regolazione di c-FLIP è una strategia sia innovativa che clinicamente traslabile per generare *ex vivo* cellule soppressorie, potenzialmente applicabili per il trattamento di patologie in cui vi è un'anormale attivazione del sistema immunitario, come le malattie autoimmuni. Inoltre c-FLIP può essere considerato un ottimo candidato come biomarcatore per semplificare la caratterizzazione la quantificazione delle MDSC nei pazienti oncologici e predirne la loro risposta alla chemioterapia

ABSTRACT

During tumor progression, cancer cells secrete many different tumor-derived factors (TDFs), like cytokines, chemokines, and metabolites, which promote the development of a flexible microenvironment inducing bot the generation of new vessels and the modification of the immune responses (Balkwill, Charles et al. 2005). Probably the most pervasive and efficient strategy of "tumor escape" relies on the tumor's ability to create a tolerant microenvironment by modification of the normal hematopoiesis. Indeed, cancers can induce the proliferation and differentiation of myeloid precursors into myeloid cells with immunosuppressive functions. These cells, named myeloid-derive suppressor cells (MDSCs), are a heterogeneous population of myeloid cells emcompassing various stages of differentiation. MDSCs prevent the activation and functionality of T lymphocytes, limiting the success of immunotherapy strategies aimed at eradicating cancer development. In this study, we demonstrated that low dose of chemotherapeutics with different molecular targets and cytotoxic action, widely used in conventional anti-cancer therapy, were able to selectively deplete monocytic-MDSCs (M-MDSCs), restoring the T cell proliferation. We also proved that these drugs exert their action through the activation of the extrinsic apoptotic death pathway and with the specific down-regulation of cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP), a well-known anti-apoptotic and drug resistance factor. In particular, recent study in mice demonstrated that the heterogeneity between the two main subsets of MDSCs, the M-MDSCs and the polymorphonuclear/granulocytic (PMN)-MDSCs, occurs from a diverse activation of the apoptotic pathways: PMN-MDSCs require the anti-apoptotic molecule MCL-1 for their development; in contrast, M-MDSC generation and survival constitutively requires the presence of c-FLIP (Haverkamp, Smith et al. 2014). Therefore, we verified the hypothesis that c-FLIP can have also a role in driving and controlling MDSC immunosuppressive properties. Here we describe that the enforced expression of c-FLIP in cells of monocytic origin is sufficient to

promote a strong immunosuppressive program, characterized by the up-regulation of several immune-related genes. c-FLIP over-expression generates human myeloid suppressors, which are potent therapeutics able to inhibit, both *in vitro* and *in vivo*, T cell activation/proliferation, and mitigate the severity of graft versus host disease (GvHD) in immunodeficient mice engrafted with human PBMCs. Moreover, the link between c-FLIP and the immunomodulatory properties was also demonstrated using M-MDSC derived from a transgenic (Tg) mouse model that express FLIP in the myeloid cell lineage. The up-regulation of c-FLIP is both original and clinically applicable as new method to generate *ex vivo* human suppressor cells for the treatment of immune-mediated disorders. Finally, c-FLIP represents an innovative biomarker that might simplify MDSC enumeration and classification, useful to define the patient's status and predict the response to chemotherapy.

INTRODUCTION

1. Emergency myelopoiesis and MDSCs.

Myelopoiesis is a structural process where a progeny of common precursor acquires specific markers and functions of circulating leucocytes, and in doing so progressively lose the ability to self-renew. Normally, physiologic numbers of mature neutrophils and monocytes are maintained by a steady-state myelopoietic pathway, whereas either acute infection, tissue damage or abnormal cellular growth (cancer) trigger the mobilization of mature neutrophils and more immature populations from the bone marrow and blood to inflammatory sites. The result of this mobilization is an early depletion of bone marrow reserves, creation of niche space, and the release of local mediators, which will drive accelerated or emergency myelopoiesis in the bone marrow. If these conditions resolve quickly, the balance of myeloid cells is restored without negative consequences for the host (Bronte, Brandau et al. 2016). Nevertheless, a great number of pathological conditions, such as chronic inflammation, autoimmune disease and cancer are associated with aberrant and sustained myelopoiesis, characterized by the accumulation of immature myeloid cells, deviated from the standard path of differentiation. Indeed, many tumor-derived factors (TDFs), cytokines, chemokines and metabolic soluble mediators promote and sustain the expansion of a heterogeneous population of myeloid cells remarkably skewed towards an immunosuppressive phenotype and endowed with regulatory functions. The appearance of this tolerogenic population, called myeloid-derived-suppressor-cells (MDSCs), represents a common trait of cancer and other diseases, such as sepsis, bacterial, viral and parasitical infectious, autoimmune diseases and aging. The immunosuppressive properties of certain subsets of myeloid cells were firstly highlighted in 1984 by Strober who originally named these cells as “natural suppressor” (NS) cells. These cells lacked typical markers of common lymphocytes, natural killer (NK) cells and macrophages but, surprisingly, showed a peculiar activity of suppressing T-cell functions. NS cells appear only briefly

during the early maturation phases of the lymphoid tissues, in the placenta during pregnancy and in neonatal maturation of lymphoid tissues, but they can be induced in adults by manipulating the lymphoid tissues with certain treatment regimens such as total lymphoid irradiation (TLI) or chemotherapy (Strober 1984). Technical limitations in purification and culture system plus the loss of a straightforward phenotype delayed the definition of the biological role and activity of NS cells. The first clear involvement of myeloid cells in controlling the immune system and consequently promoting tumor growth was provided in 1995. Seung and colleagues demonstrated that the administration of the antibody against the antigen Gr-1 to immunocompetent mice reduced the growth of UV light-induced tumor (Seung, Rowley et al. 1995). At the beginning, this unexpected result was attributed to the elimination of $Gr\text{-}1^+$ granulocytes, but successive reports proved that $Gr\text{-}1^+$ cells were mostly $CD11b^+$ and comprised cells at different maturation stages, both polymorphonuclear and monocyte-like (Kusmartsev and Gabrilovich 2006, Serafini, Borrello et al. 2006). For many years the heterogeneity of the $CD11b^+/Gr\text{-}1^+$ cells and the use of several acronyms to define them generated some misunderstanding among different groups of investigators. In an attempt to codify analysis of the nature and clinical significance of these cells, in 2007 a board of investigators agreed to use the common term "MDSC", highlighting their myeloid origin, their immune-suppressive function and the systemic expansion of these cells in cancer-related context.

1.1. Mouse MDSCs.

MDSCs are characterized by their flexible and peculiar composition for every disease scenario and often change following the kinetics and development of the disease. In mice, historically, MDSCs were defined as cells expressing both Gr-1 and CD11b (also known as integrin αM) markers. Nevertheless, recent studies have led to the identification of two main subsets with different phenotypic and biological properties: monocytic (M)-MDSCs and polymorphonuclear

/granulocytic (PMN)-MDSCs. Both the subpopulations share the CD11b myeloid marker but can be easily distinguished by the different expression of the two main Gr-1 epitopes, Ly6C and Ly6G. M-MDSCs ($Gr\text{-}1^{lo/int}CD11b^{+}Ly6C^{hi}Ly6G^{-}$) display the highest immunosuppressive activity in an antigen-non-specific manner, whereas PMN-MDSCs ($Gr\text{-}1^{hi}CD11b^{+}Ly6C^{lo}Ly6G^{+}$) are less immunosuppressive and exert their function by antigen-specific mechanisms (Figure 1A). M-MDSCs are side scatter low (SSC^{lo}), while PMN-MDSCs are side scatter high (SSC^{hi}). M-MDSCs usually express higher levels of F4/80 (macrophage marker), CD115 (c-Fms, the receptor for M-CSF/CSF-1), CD124 (receptor α of IL-4), and CCR2 (receptor for monocytes chemoattractant protein, also known as CCL2), although these markers are not uniformly expressed by MDSCs induced by all tumors. Moreover, M-MDSCs, but not PMN-MDSCs, when cultured with granulocyte macrophage colony-stimulating factor (GM-CSF), mature *in vitro* and acquire F4/80 and CD11c expression (Youn, Nagaraj et al. 2008, Dolcetti, Peranzoni et al. 2010). MDSCs express variable amounts of markers associated with early stages of myeloid differentiation (CD31 and ER-MP58), low levels of the major histocompatibily complex (MHC) class II and co-stimulatory molecules (e.g., CD80), in line with their origin from immature myelo-monocytic precursors (Bronte, Apolloni et al. 2000), as well as the co-inhibitory molecule programmed death ligand-1 (PDL-1) (Youn, Nagaraj et al. 2008), which was recently associated with MDSC-dependent impairment of T cell effector functions. In many tumor models, as well as in cancer patients, PMN-MDSCs are the predominant subset, representing 70 to 80% of the tumor-induced MDSCs, compared to 20 to 30% of the cells reflecting the monocytic lineage (Gabrilovich, Ostrand-Rosenberg et al. 2012). Besides the phenotypical characterization, what really defines MDSCs is their immunoregulatory activity. This property mainly relies on the activity of enzymes like arginase 1 (ARG1), indoleamine-2,3-dioxygenase 1 (IDO1) and nitric oxide synthase 2 (NOS2/iNOS) (Zoso, Mazza et al. 2014). The two sub-populations also differ in the effector pathways used to suppress T-cell activation. M-MDSCs suppress $CD8^{+}$ T-lymphocytes proliferation mainly through activation of iNOS and ARG1 enzymes and through the production of reactive nitrogen species (RNS). PMN-MDSCs, on

the contrary, can express some levels of ARG1 but suppress mainly through the release of reactive oxygen species (ROS). A relevant number of M-MDSCs, in tumor-bearing mice, acquires phenotypic, morphological and functional features of PMN-MDSCs by a mechanism that involves the epigenetic downregulation of the retinoblastoma protein (Rb1) by histone deacetylases (Youn, Kumar et al. 2013). Thus, M-MDSCs not only have the capacity to strongly down-modulate antitumor immunity but also serve as "precursors" that maintain the PMN-MDSC pool. M-MDSCs proliferate faster than either PMN-MDSCs or the normal monocytes, form colonies in agar, and can generate a wide range of myeloid cells when adoptively transferred to tumor-bearing host (Ugel, Peranzoni et al. 2012). By preventing the extrinsic apoptotic death pathway and the activation of caspase-8, the cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP) is constitutively required for the development of M-MDSCs, whereas myeloid cell leukemia 1 (MCL-1) protein, which controls the intrinsic mitochondrial death pathway, is essential for the development of PMN-MDSCs (Haverkamp, Smith et al. 2014). The plasticity of MDSCs relies on the ability of these myeloid cells to lose their lineage identity in response to specific environmental signals. MDSC recruitment into the tumor side is mainly mediated by the CCR2/CCL2 axis allowing to maintain the presence and frequency of non-proliferating tumor-associated macrophages (TAMs) population (Movahedi, Laoui et al. 2010). Indeed, TDFs can induce and promote the tumor-infiltrating MDSC differentiation in TAMs and tumor-associated-neutrophils (TANs).

1.2. Human MDSCs.

In human, the first observations of the presence of immature cells endowed with immunosuppressive ability were reported in 1995 in head and neck cancer tissues and, initially, these cells were defined as tumor-infiltrating and blood circulating $CD34^+$ cells, amplified by the tumor-released GM-CSF (Pak, Wright et al. 1995). To further support the role of enhanced human myelopoises in generating regulatory cells, treatment with granulocytes colony stimulating factors (G-CSF)

and GM-CSF for autologous stem cells transplantation was shown to induce suppressive $CD14^+$ able to cause $CD4^+$ and $CD8^+$ T cell apoptosis, resulting in inhibition of T cell function (Ino, Singh et al. 1997). All these studies supported the idea that a myeloid population of monocytic lineage, under specific stimuli, might become suppressive and a great similarity between these cells and murine MDSCs was noted. Nowadays, CD34 marker is no longer considered a universal marker of immunosuppressive cells. These studies also underline the substantial heterogeneity of human MDSCs in terms of surface markers presence and expression level. The phenotyping of human MDSCs is complicated by the fact that Gr-1 is not expressed on human leukocytes. Multiple human MDSC subsets with different phenotypes have been documented in several types of tumors in the last 20 years (Solito, Marigo et al. 2014). All reported phenotypes are defined on the basis of a combination of myeloid markers and actually suffer from the lack of a specific marker. The initial assumption that MDSCs are solely constituted of immature myeloid cells (Almand, Clark et al. 2001, Mirza, Fishman et al. 2006), is now challenged by reports describing MDSCs with morphology and phenotype consistent with cells possessing more differentiated features. After more than 20 years of intensive study, scientists in the field agreed upon a multiparametric flow cytometry assay for the MDSC characterization and monitoring, which allows the simultaneous detection of different human subsets (Mandruzzato, Brandau et al. 2016). Similarly to what previously described in mice, M-MDSCs share the morphology of monocytes and are characterized by the expression of CD14 and lack of CD15 markers (Filipazzi, Valenti et al. 2007) (Mandruzzato, Solito et al. 2009); PMN-MDSCs contain a cell population resembling granulocytes and are phenotypically characterized by CD15 and CD66b expression and the absence of CD14 (Schmielau and Finn 2001, Rodriguez, Ernstoff et al. 2009, Brandau, Trellakis et al. 2011). Finally, immature MDSCs (I)-MDSCs are cells with immaturity characteristics, defined essentially as lineage negative cells, which mouse counterpart is yet to be identified (Diaz-Montero, Salem et al. 2009) (Solito, Falisi et al. 2011) (Figure 1B). A useful marker for the identification of immunesuppressive MDSCs is the CD124; in fact, its expression on MDSCs of colon cancer and melanoma patients correlated with a more immunosuppressive

phenotype (Mandruzzato, Solito et al. 2009). Human MDSCs have been well characterized in a long list of solid tumors: breast cancer, non-small cell lung cancer, colon and colorectal cancer, sarcoma, gall bladder, melanoma (Diaz-Montero, Salem et al. 2009), head and neck squamous carcinoma (Almand, Clark et al. 2001), carcinoid, renal cell carcinoma (RCC, (Rodriguez, Ernstoff et al. 2009), gastrointestinal cancer, esophageal cancer (Gabitass, Annels et al. 2011), bladder cancer (Eruslanov, Neuberger et al. 2012), urothelial cancer (Brandau, Trellakis et al. 2011). MDSCs were also detected in different hematological malignancies, such as non-Hodgkin's lymphoma and multiple myeloma (Solito, Marigo et al. 2014). Nevertheless, it remains very difficult to clearly identify MDSC subsets in cancer patients. PMN-MDSCs are very cryosensitive (Trellakis, Bruderek et al. 2013), thus functional studies can be performed only on fresh samples. PMN-MDSCs can contaminate peripheral blood mononuclear cells (PBMCs) separation, as previously described for renal cell cancer (RCC) where patients' PMN-MDSCs suppressed the proliferation of T cells by ARG1-mediated CD3 ζ-chain down-regulation (Zea, Rodriguez et al. 2005). Moreover, patients with colon and lung cancers display increased levels of PMN-MDSCs in blood as well (Mandruzzato, Solito et al. 2009) (Liu, Wang et al. 2010). Circulating M-MDSCs ($CD11b^{+}Lin^{-}CD33^{+}HLA\text{-}DR^{-}CD14^{+}$) and Treg are increased in metastatic prostatic cancer patients compared to healthy donors and negatively correlate with patients survival. Furthermore, the same study showed that patient MDSCs isolated from peripheral blood have immune suppressive capabilities on activated T cells, probably through up-regulation of iNOS (Idorn, Kollgaard et al. 2014). Also M-MDSCs are present and detectable in the peripheral blood of melanoma, hepatocellular carcinoma, and head and neck cancer patients (Hoechst, Ormandy et al. 2008). Collectively, all these studies indicate that expansion of MDSCs in cancer patients is a general phenomenon accompanying tumor progression. Circulating MDSC levels correlated with response to therapy (Liu, Wang et al. 2010, Huang, Zhang et al. 2013) and surgery (Yuan, Zhao et al. 2011) and the analysis of the clinical outcome of cancer patients revealed that MDSC frequency in blood is associated with prognosis and clinical outcome (Gabitass, Annels et al. 2011).

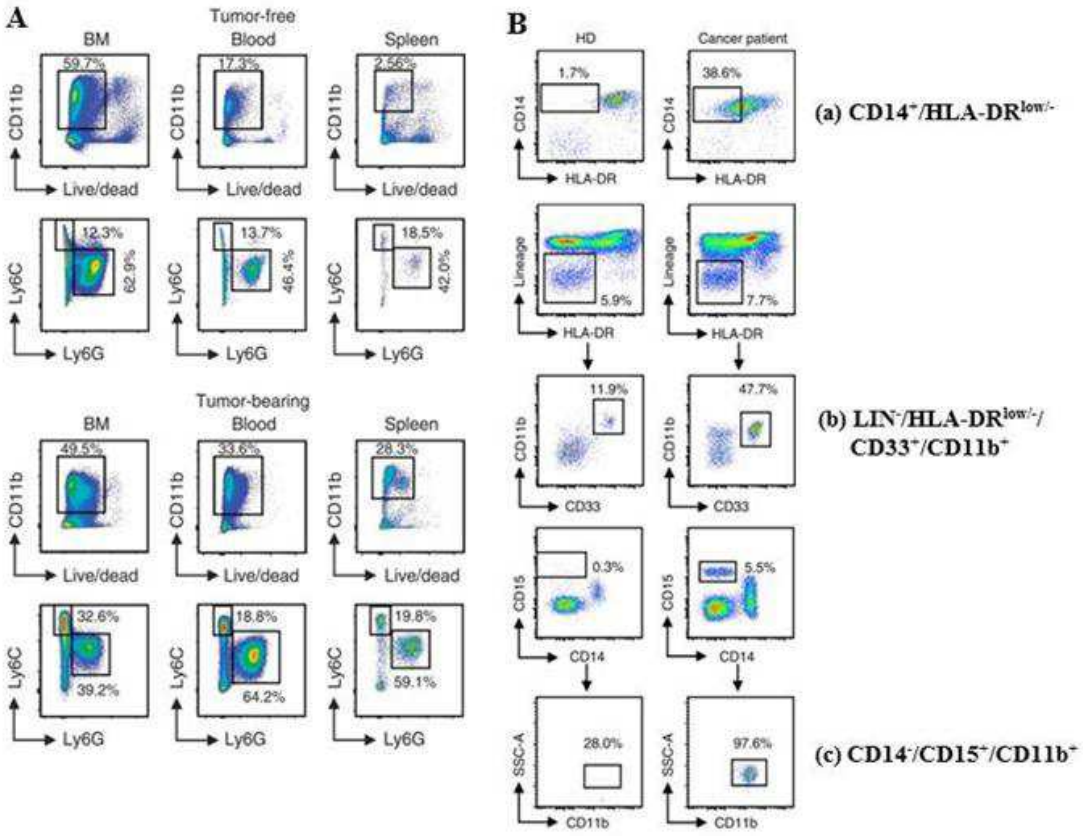

Figure 1. Gating strategy for the identification of mouse and human MDSC subsets. A) Gating strategy used to define MDSC subpopulations in BM, blood and spleen of C57Bl/6 tumor-free or MCA203 tumor-bearing mice. After exclusion of doublets (not shown), live CD11b$^+$ cells were gated and the proportion of Ly6C and Ly6G cells was evaluated. **B)** Gating strategy for the identification of MDSC subsets in the peripheral blood of healthy donors (HD) and melanoma patients. Doublets were excluded and live PBMC were gated (not shown). (a) CD14$^+$HLA-DR$^{-/lo}$ M-MDSC. Monocytes were gated on the basis of FSC and SSC parameters and HLA-DR downregulation was defined by FMO control. (b) Lin$^-$HLA-DR$^-$CD33$^+$ I-MDSC. (c) CD14$^-$CD15$^+$CD11b$^+$ PMN-MDSC.
Adapted from Bronte V., 2016

1.3. MDSC immunosuppressive mechanisms.

The ability of MDSCs to control the immune responses and block T cell activity is now considered one of the major immunological hallmarks of cancer (Cavallo, De Giovanni et al. 2011, Hanahan and Weinberg 2011, Condamine, Ramachandran et al. 2015). MDSCs inhibit immune suppressive functions with 4 different mechanisms: (a) depletion of essential metabolites, (b) production of reactive oxygen species, (c) interfering with T cells migration and viability and (d) T regulatory lymphocyte (Treg) induction and M2 macrophage reprogramming.

a) Depletion of essential metabolites. Arginine, tryptophan and cysteine are essential amino acids for T cells fitness and metabolism. L-Arginine represents the common substrate for two enzymes: iNOS and ARG1. iNOS generates nitric oxide (NO), ARG1 converts L-arginine to urea and L-ornithine. MDSCs express high levels of both enzymes. It is well established that NO inhibit the T-cell signaling cascade downstream the IL-2 receptor, blocking the phosphorylation and activation of Janus protein tyrosine kinases (JAK) 3 and signal transducer and activator of transcription (STAT) 5 transcription factors, down-regulating MHCII gene expression and inducing T cell apoptosis. Moreover, high concentrations of NO exert a direct pro-apoptotic effect on T cells, induced by the accumulation of the tumor suppressor protein p53 by CD95 and TNF receptors family members. ARG1 activity causes the depletion of L-arginine and the translational blockade of the ζ chain of CD3, preventing T cells responses. L-Arginine starvation blocks protein translation through the accumulation of empty aminoacyl tRNA that activates the kinase general control non-derepressible 2 (GCN2) and phosphorylates the translational of the eukaryotic initiation factor 2 alfa (eIF2α) for the isoform beta (eIF2β), thus interfering with protein synthesis. Another amino acid involved in T cell function and immune regulation is tryptophan. This amino acid degradation is catalyzed by the 2 isoenzymes of IDO enzyme, IDO1 and IDO2. These enzymes are expressed by tumor cells and specific leukocyte subsets such as TAMs, dendritic cells (DCs),

and MDSCs (Srivastava, Sinha et al. 2010) and are involved in regulation of local inflammation. In particular, the 2 enzymes catalyze the degradation of the amino acid along the kynurenine pathway. L-trypthophan starvation activates GCN2 kinase, which in turns inhibits $CD8^+$ T cell proliferation, causing cell cycle arrest and inducing anergy and directs $CD4^+$ T cell differentiation towards a Treg phenotype by the forkhead box P3 (FoxP3) transcription factor upregulation (Munn, Sharma et al. 2005); even if these findings are now challenged by the demonstration that GCN2 is dispensable for T cells fitness independently of environmental amino acid sensing (Van de Velde, Guo et al. 2016). Finally, MDSCs compete with antigen presenting cells (APC), such as macrophages and DCs, for cystine import and limit the availability of cysteine in the microenvironment. T lymphocytes proliferation and fitness rely on the availability of L-cysteine but they lack both the enzymes to import it, thus depending on APC, during the immunologic synapsis. Consequently, T cells display impaired activation and function in a MDSC-conditioned, poor cysteine environment (Rolinski and Hus 2014).

b) Production of reactive oxygen species. ARG1 and NOS activities not only have a direct negative effect on T cells, but also lead to an increased production of highly reactive radical compounds by MDSCs, such as ROS and RNS, all species that have a high reactivity for macromolecules like DNA, lipids and proteins. MDSCs produce a high amount of ROS, such as hydrogen peroxide (H_2O_2), which affects T cell fitness by down-regulating CD3 ζ-chain expression and reducing cytokine secretion, as observed in pancreatic cancer and in melanoma (Otsuji, Kimura et al. 1996, Schmielau and Finn 2001). At high ROS concentration, radicals can directly react with macromolecules or combine with NO to generate more dangerous RNS, such as peroxynitrite and dinitrogen trioxide, which can avoid the detoxifying system and nitrate/nitrosylate tyrosine, cysteine, methionine and tryptophan in different proteins and enzymes, thus changing their biological functions. Under pathological conditions (i.e. tumor), RNS may induce apoptosis and autophagy directing tumor evolution, and more

importantly suppress T cell trafficking and cytotoxic functions contributing in shaping an immune privileged environment that promotes tumor outgrowth. RNS indeed can prevent antigen-specific activation of $CD8^+$ T cells, altering the immunodominant peptide structure, the peptide loading process on MHC-I on target cells, the receptor of T cells (TCR) binding to peptide-MHC-I complex (Hardy, Wick et al. 2008) or the TCR signaling ability (Nagaraj, Schrum et al. 2010). RNS can act on α and β chain of the TCR and modify leukocytes trafficking promoting homing to tumor of immune suppressive subsets other than T cells. This is in part mediated by tyrosine nitration of either chemokines (CCL2, CCL5, CCL21, CXCL12) or receptors (CXCR4) (Molon, Ugel et al. 2011, De Sanctis, Sandri et al. 2014).

c) Interfering with T cells migration and viability. To exert their functions, T cells require trafficking to lymph nodes and to tumor site and this process represents a target for MDSC inhibitory functions. Thanks to the activity of the metallo-proteinase ADAM17, MDSCs cleave the L-selectine (CD62L), which is a homing receptor critical for directing naïve T cells to lymph nodes. Moreover, the RNS produced by MDSCs modify the CCL2/CCR2 axis by nitrosylation of CCL2. As a result, only MDSCs but not cytotoxic T lymphocytes (TLs) are recruited at the tumor (Molon, Ugel et al. 2011).

d) Treg induction and M2-macrophage reprogramming. In the tumor microenvironment, MDSCs produce high amount of transforming growth factor-β (TGF-β) and interleukin (IL)-10. TGF-β arrests T lymphocytes cell cycle, blocks the differentiation of $CD4^+$ T cells in Th1 or Th2 cells and promotes the clonal expansion of antigen-specific natural Treg cells inducing the conversion of naïve $CD4^+$ T cells into induced (i) Treg cells in combination with other products, such as interferon (IFN)-γ and IL-10 or retinoic acid (Serafini, Mgebroff et al. 2008, Hoechst, Gamrekelashvili et al. 2011). Moreover, by decreasing macrophage production of IL-12,

MDSCs skew macrophages towards an M2 phenotype (Sinha, Clements et al. 2007). Thus MDSCs are also able to indirectly promote immune suppression, favoring the generation or the expansion of other regulatory populations (Figure 2).

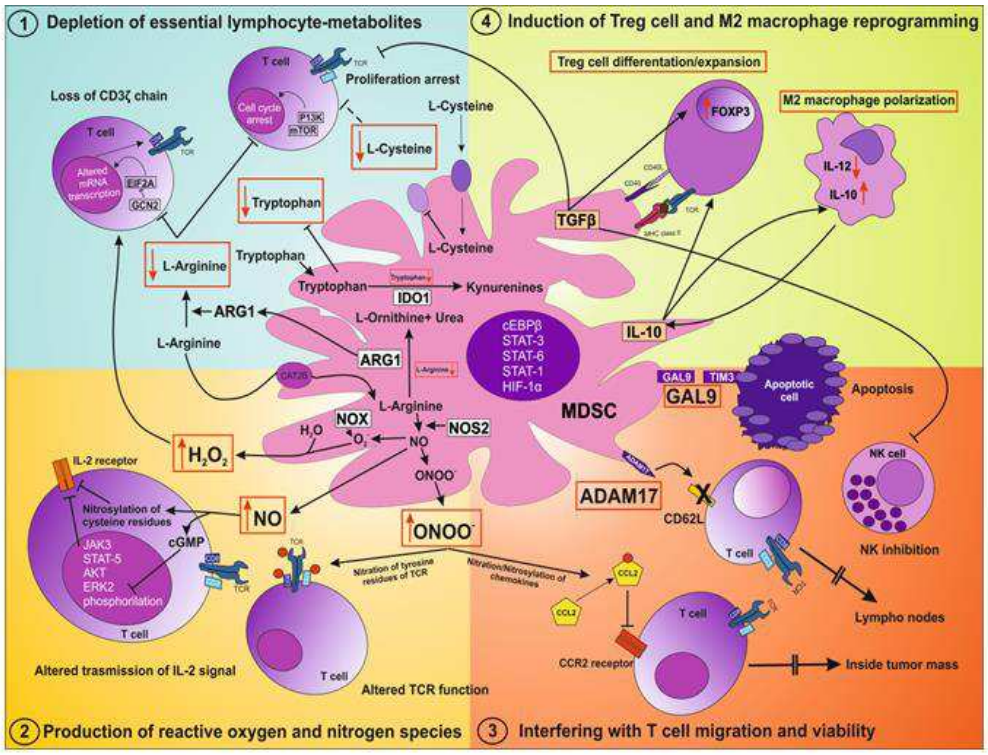

Figure 2. Immune suppressive functions of MDSCs. MDSCs inhibit the immune responses by four main mechanisms. **(1)** MDSCs deplete essential metabolites for T lymphocyte fitness: l-cysteine (through its altered transport), l-tryptophan (by the activation of IDO1) and l-arginine (by the activation of both ARG1 and NOS2). The final outcome of nutrient deprivation is T cell proliferation arrest. The depletion of l-arginine by ARG1 activity also induces the translational blockade of the ζ chain of CD3 that prevents T cells from responding to various stimuli. The production of NO inhibits the signaling cascade downstream of the IL-2 receptor by different mechanisms (green panel). **(2)** High arginase and NOX2 activities in combination with increased NO production by the MDSCs lead to an increased production in ROS and RNS. High amounts of ROS affect T cells by down-regulating CD3 ζ chain expression and reducing cytokine secretion. RNS can act on the α and β TCR chains preventing TCR signaling and promoting dissociation of CD3 ζ chain from the complex. RNS also modifies trafficking of leukocytes promoting homing of immune suppressive subsets other than T cells by nitration/nitrosylation of chemokines (CCL2, CCL5, CCL21, CXCL12) or receptors (CXCR4) (red panel). **(3)** MDSCs interfere with T cell migration and viability; in fact, MDSCs express the metalloproteinase ADAM17 able to cut the integrin CD62L on the membrane of T cells; moreover, MDSCs express PDL-1, which that binds PD-1 on T cells, promoting their inactivation. Finally, the TGF-β release by MDSCs can promote NK cell inhibition (blue panel). **(4)** MDSCs drive the differentiation of specific subsets of regulatory cells: by TGF-β release, MDSCs promote the clonal expansion of antigen-specific natural Treg cells and induce the conversion of naïve CD4 T cells into induced Treg cells. MDSCs skew macrophages towards an M2 phenotype by releasing IL-10 (orange panel). *Adapted from De Sanctis F., et al., 2016*

1.4. MDSC-induced mechanism of tumor promotion.

MDSCs not only play a role in creating an immunosuppressive microenvironment but can also favor tumor growth and metastatic spread by several other mechanisms. Firstly, MDSCs can protect tumor cells from immune-mediated killing; this mechanism was shown to be important in the increased permissiveness for metastasis seen in pregnancy. Specifically, decreased natural killer (NK) cell functionality in pregnant mice was dependent on MDSC accumulation (Mauti, Le Bitoux et al. 2011). MDSCs are capable of supporting tumor growth through remodeling the tumor microenvironment (Sevko and Umansky 2013, Ortiz, Lu et al. 2014). They have been shown to produce vascular endothelial growth factor (VEGF), basic fibroblast growth factor (Bfgf), prokineticin-2 (Bv8, a VEGF analogue) and MMP9, all essential mediators of neoangiogenesis and tissue invasion at the tumor site. The expression of these molecules has been linked to MDSC-mediated metastasis and is independent of their immunosuppressive capacity (Casella, Feccia et al. 2003, Tartour, Pere et al. 2011). The situation of a pre-metastatic niche, in which non-cancer, bone marrow cells promote future metastasis, preparing distant organs for the arrival of tumor cells might also involve MDSC population (Kaplan, Psaila et al. 2006). Mouse models allowed to demonstrate that MDSCs appear in the lungs two weeks prior to the appearance of metastases and the presence of these cells in the lungs correlated with decreased immune function of these tissues. The hypoxia, condition of the tumor microenvironment, induces VEGF, S100A8/A9, IL-6, and IL-10 (via activation of the sphingosine-1-phosphate receptor 1 (S1PR1)/STAT3 axis in the lungs), all mediators of the recruitment and activation of MDSCs in the lungs and of the establishment of the premetastatic niche (Hiratsuka, Watanabe et al. 2006, Erler, Bennewith et al. 2009, Deng, Liu et al. 2012). MDSCs can assist the metastatic process also by inducing the epithelial-to-mesenchymal transition (EMT), a situation in which cells acquire improved spreading skills. In the ret model of spontaneous mouse melanoma, $CD11b^{+}Gr1^{+}$ cells were shown to infiltrate primary tumors in a mechanism that was dependent on CXCL5.

Depletion of these cells resulted in smaller, less nodal tumors and a drastic reduction in metastasis number. *In vitro* co-culture experiments showed that these cells induced a stem-like phenotype in tumor cells and that inhibition of the TGF-β, epidermal growth factor (EGF), and/or hepatocyte growth factor (HGF) pathways could reverse this effect (Toh, Wang et al. 2011). *Bona fide* MDSCs are not produced in healthy individuals. Even in the early stages of cancer, cells with an MDSC-like phenotype may not have immunosuppressive activity (Eruslanov, Bhojnagarwala et al. 2014). Recent evidence have linked accumulation of immature myeloid cells with an MDSC-like phenotype during chronic inflammation with the early stages of tumor development. Exposure of mice to cigarette smoke caused accumulation of these cells in lung and spleen; however, these cells became immunosuppressive MDSCs only after the development of lung cancer (Ortiz, Lu et al. 2014). Nevertheless, their depletion increased survival. In a model of skin carcinogenesis, accumulation of immature myeloid cells without suppressive function in the skin of mice strongly promoted tumor formation that correlates with the CCL4-mediated recruitment of IL-17–producing $CD4^+$ T cells (Ortiz, Kumar et al. 2015). $CD11b^+Gr1^+$ cells were also recently shown to control cellular senescence promoting a more aggressive disease in a mouse model of spontaneous prostate cancer trough limiting the IL-1α–mediated senescence (Di Mitri, Toso et al. 2014). Another mechanism of MDSC-dependent tumor senescence inhibition was described in human ovarian cancer (Cui, Kryczek et al. 2013). The C-terminal-binding protein 2, also known as CtBP2, is a transcription corepressor recruiting both histone deacetylases, methylases and demethylases on target genes, which in turn remodels chromatin condensation and gene expression. CtBP2 modulates the expression of genes involved in sphere formation in ovarian primary tumor cells. MDSCs break this equilibrium and promote cancer stemness and metastasis through induction of microRNA101 in tumor cells. The microRNA101 targeting CtBP2 mRNA induces the expression of genes involved in sphere formation such as the octamer-binding transcription factor 4 (OCT3/4), the sex determining region Y-box 2 (SOX2) and NANOG. Accordingly, dense MDSC infiltrate, high microRNA101 expression and low CtBP2 levels correlated in ovarian cancer patients with a worse clinical outcome

(Cui, Kryczek et al. 2013). Thus, cells with an MDSC-like phenotype play a significant role in tumor development and progression via mechanisms that are not necessarily related to their ability to suppress tumor-specific immune

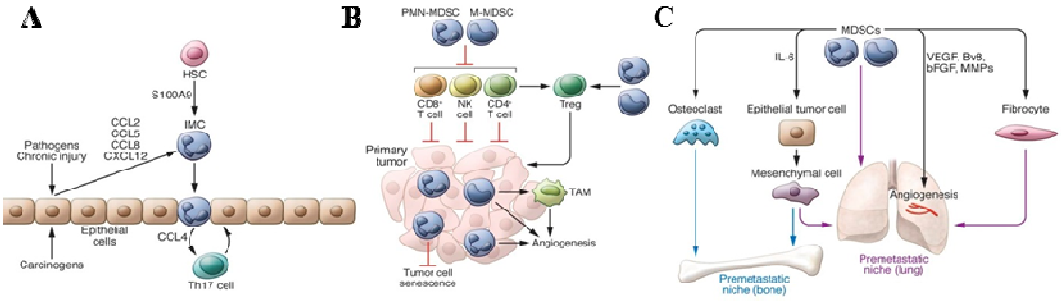

Figure 3. Potential role of immature myeloid cells and MDSCs in the regulation of tumor development and progression. A) I-MDSCs are produced in response to inflammatory stimuli. However, these cells often lack immunosuppressive activity. They contribute to tumorigenesis by recruiting proinflammatory CD4+ T cells that promote epithelial cell proliferation. **B)** Tumor development is associated with the expansion of cells with acquired immunosuppressive activity (MDSCs). These cells also promote tumor cell invasion and angiogenesis and neutralize tumor cell senescence. **C)** In metastatic tumors, MDSCs, in addition to promoting tumor cell invasion and angiogenesis, can support EMT and differentiation of osteoclasts supporting bone resorption.
Adapted from Marvel D et al., 2015

responses (Figure 3).

1.5. Signaling pathways regulating MDSC ontogenesis and function.

There is a strong interest in elucidating the role of signaling pathways involved in the regulation of MDSC function. Most signaling pathways lead to activation of transcription factors and genes that are involved in cell proliferation, survival, and differentiation. Therefore, it is imperative to examine the role of cell cycle regulators during MDSC development and function. The analysis of gene expression arrays from tumor MDSCs showed a significant alteration in the expression of genes involved in cell cycle control. It is widely believed that perturbation of signaling pathways that are required for myeloid development leads to the generation of MDSCs. Ras/MAPK, PI3K/Akt, Jak/Stat, TGFb, CCAAT-enhancer-binding proteins (C/EBPβ) and Toll-LIKE receptors (TLRs) signaling pathways together with the cytokines M-CSF, GM-CSF, G-CSF and

micro RNAs (miRs) are involved in the regulation of various aspects of MDSC biology. Members of the STAT family (STAT1, STAT3, STAT5 and STAT6) play a crucial role in the polarization of myeloid cells functions. STATs are phosphorylated by members of JAK families; they dimerize and are translocated into the nucleus where they modulate the expression of target genes. STAT3 is one of the master transcriptional factors of MDSC expansion and its genetic ablation promotes a substantial MDSC contraction in tumor-bearing mice (Kortylewski, Kujawski et al. 2005). STAT3 not only promotes MDSC survival by inducing the expression of cyclin D1, myc and B-cell lymphoma XL (BCL-XL), but it also increases the MDSC production of ROS by phagocytic oxidase (Corzo, Cotter et al. 2009). STAT1 is the main transcription factor downstream IFN-γ and IL-1β signaling cascade and controls important genes for MDSC activity since STAT1-deficient MDSCs are unable to inhibit T cell activation due to the defective iNOS and ARG1 up-regulation (Kusmartsev and Gabrilovich 2005). STAT-1 is involved in the iNOS induction mediated by interferon regulatory factor (IRF)-1. STAT5, one of the main factors activated by GM-CSF in the myeloid lineage, also contributes to regulate MDSC survival (Xin, Zhang et al. 2009). The activation of STAT6 as well as STAT1 induces the up-regulation of ARG1 and iNOS and leads to the increased production of suppressive cytokines, such as TGF-β (Takaku, Terabe et al. 2010). Another relevant transcription factor for the differentiation of the myeloid lineage and a master switch for "emergency granulopoiesis" is C/EBPβ (Hirai, Zhang et al. 2006, Manz and Boettcher 2014). C/EBPβ activates several genes, including c-myc, IL-6, and the gene encoding for the common signaling β-chain receptor that regulates the signal transduction for GM-CSF, IL-3, and IL-5 cytokines (van Dijk, Baltus et al. 1999). The importance of C/EBPβ in MDSC differentiation is demonstrated by the lack of their accumulation in the spleen of tumor-bearing, C/EBP$\beta^{-/-}$ mice and the failure of C/EBPβ-deficient bone marrow (BM) cells to differentiate *in vitro* into functionally competent MDSCs (Marigo, Bosio et al. 2010). Interestingly, a direct link between STAT3 and C/EBPβ exists: STAT3 transduces G-CSF signal and induces C/EPBβ expression in myeloid progenitor cells (Zhang, Nguyen-Jackson et al. 2010). Moreover, C/EPBβ can be regulated by orphan nuclear receptor

retinoic-acid-related receptor gamma full length protein (RORC1), promoting emergency granulo-monocytopoiesis (Strauss, Sangaletti et al. 2015). Also the TLR family plays an important role in myeloid cells in the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, primarily through the myeloid differentiation primary response gene 88 (MyD88), and myelopoiesis during tumor progression (Hong, Chang et al. 2013). Recently, phospholipase C gamma 2 (PLCγ2) and src homology 2 domain-containing inositol 5′-phosphatase-1 (SHIP-1), two enzymes involved in physiologic hematopoiesis, have been reported to be potential MDSC regulators; in fact, their ablation promotes MDSC accumulation and their immune suppressive properties in tumor-bearing mice, suggesting that they negatively regulate MDSC biology (Pilon-Thomas, Nelson et al. 2011, Capietto, Kim et al. 2013). Finally, different miRs are either up- or down-regulated during MDSC differentiation. GM-CSF- and IL-6-mediated MDSC proliferation and differentiation required up regulation of miR-21 and miR-155 (Li, Zhang et al. 2014). On the contrary, miR-142-3p must be repressed to allow acquisition of immune suppressive properties in MDSCs; interestingly, miR-142-3p controls the expression of the gp130 signaling subunit of the IL-6 receptor complex, the downstream STAT3 phosphorylation, and the balance between different isoforms of C/EBPβ (Sonda, Simonato et al. 2013).

1.6 Strategies to therapeutically target MDSCs.

Since MDSCs fuel one of the main immunosuppressive circuits in cancer, several pharmacological approaches, which involve either MDSC elimination or modulation of their functions, are currently being explored in tumor-bearing hosts. Moreover, these novel approaches could be potentially translated to the therapy of other diseases in which MDSCs can play a pathogenic role, such as immunosuppression/immune deviation associated with chronic infections. For simplicity, we can divide these MDSC inhibitors in four classes according to their ability to control: firstly, MDSC immune regulatory properties; secondly, MDSC development; thirdly, MDSC differentiation; and finally, MDSC depletion.

Targeting MDSC immune regulatory properties. MDSCs can be functionally inactivated by targeting their suppressive machinery and, at the moment, several approaches have been already exploited. Both ARG1, iNOS and IL-4Rα expression have been shown to be downregulated in response to phosphodiesterase-5 (PDE-5) inhibition (Serafini, Borrello et al. 2006). A clinical trial (number NCT00894413) with tadalafil, an inhibitor of PDE-5, evaluated the effect of this treatment on immune function in patients with head and neck squamous cell carcinoma (HNSCC) and reported that both ARG1 and iNOS activities were significantly reduced in tadalafil-treated patients. Moreover, this pharmaceutical treatment promoted the contraction of both circulating and tumor-infiltrating $CD33^{+}HLA\text{-}DR^{-}IL\text{-}4R\alpha^{+}$ M-MDSCs in treated patients (Califano, Khan et al. 2015). In addition, the administration of tadalafil to a patient with end-stage relapsed/refractory multiple myeloma was able to strongly reduce MDSC functions favoring a durable anti-myeloma immune and clinical response (Noonan, Ghosh et al. 2014). Other clinical compounds, able to inhibit iNOS, are now under investigation. For example, the use of nitro-aspirin, a pharmacological compound able to inhibit iNOS activity affecting NO release, was described to normalize the immune status of orally treated, tumor-bearing mice improving tumor-antigen-specific T responses and enhancing the preventive and therapeutic effectiveness of the antitumor immunity elicited by cancer vaccination (De Santo, Serafini et al. 2005). Interestingly, [3-(aminocarbonyl)furoxan-4-yl] methylsalicylate (AT38), another NO-donating compound was shown to decrease MDSC inhibitory activity by reducing the nitration of chemoattractants, such as CCL2 and CXCL12 chemokines, restoring the T cell ability to migrate within tumor primary lesion. Indeed, the administration of this drug significantly reduced the expression of both iNOS and ARG1 enzymes in myeloid cells, preventing RNS generation within the tumor environment (Molon, Ugel et al. 2011). Finally, inhibition of COX-2 decreased the production of immunosuppressive prostaglandin E2, limiting cancer progression. Celexobib, the COX2 inhibitor, supplied in combination with DCs pulsed with tumor lysates improved the survival of mesothelioma-bearing mice (Veltman, Lambers et al. 2010).

Inducing MDSC depletion. Accumulating evidence indicates that the antitumor activity of chemotherapy also relies on several off-target effects, especially directed at the host immune system, that cooperate for successful tumor eradication (Bracci, Schiavoni et al. 2014). In particular, some conventional chemotherapy agents, such as gemcitabine and 5-fluouracil (5-FU), showed a highly effective cytotoxic action on MDSCs. In more details, gemcitabine, an antimetabolite drug (nucleoside analogue), used for the treatment of pancreatic, breast, ovarian, and lung cancers, was reported to deplete MDSCs in tumor-bearing mice, resulting in enhanced antitumor immunity (Suzuki, Kapoor et al. 2005, Le, Graham et al. 2009, Tomihara, Fuse et al. 2014). Another antimetabolite, 5-FU, used at low doses, was also shown to induce MDSC apoptosis (Vincent, Mignot et al. 2010). Interestingly, MDSCs are more sensitive to these molecules than other immune cells or tumor cells. This dominant effect was explained by a lower expression of thymidylate synthase by MDSCs. The DNA-demethylating agent 5-azacytidine may also reduce the accumulation and function of MDSCs induced in the mouse TC-1/A9 and TRAMP-C2 tumor models (Mikyskova, Indrova et al. 2014). Docetaxel, (a mitotic inhibitor, semisynthetic analogue of paclitaxel) was shown to impair MDSC-suppressive function, predominantly by blocking STAT3 phosphorylation and promoting MDSC differentiation into M1 macrophages (Kodumudi, Woan et al. 2010). Similarly, low-dose of paclitaxel promotes MDSC differentiation into DCs *in vitro (*Michels, Shurin et al. 2012). The anthracycline doxorubicin has been described for its plethoric immunostimulatory effects and it was recently demonstrated, in different mouse cancer models, that this drug selectively, but however transiently, eliminates and inactivates MDSCs (Alizadeh and Larmonier 2014). This preferential targeting of MDSCs translates into increased effector lymphocyte to immunosuppressive MDSC ratios and is associated with enhanced $CD4^+$, $CD8^+$, and NK cell activation and pro-inflammatory cytokine production, that are fundamental prerequisites to establish a therapeutic effect mediated by cancer immunotherapy. For example, a chemo-immunotherapeutic regimen based on the association of different chemotherapies with adoptive cell transfer (ACT) of antigen-specific $CD8^+$ T lymphocytes was described to restrain tumor

development and improve the overall survival of tumor-bearing mice since the chemotherapeutic treatment selectively eliminated MDSCs (Ugel, Peranzoni et al. 2012).

Targeting MDSC development. Another attractive strategy is based on neutralizing the factors that are involved in MDSC expansion from the hematopoietic precursors, thus compromising their development. Although promising, this task is made complex by the plethora of TDFs involved in MDSC expansion and recruitment. Some of these factors, however, trigger a common intracellular pathway in MDSCs that involves the STAT family of transcription factors. Among the members of this family, STAT3 is well recognized as a key regulator of MDSC biology. MDSCs are characterized by a persistent STAT3 activation induced by various alterations in tumor microenvironment, including oncogenic activation of receptor tyrosine kinases and release of IL-6, VEGF, and IL-10. The consequences of STAT3 constitutive activation is the up-regulation of the numerous STAT3-dependent genes, among which there are anti-apoptotic ($BCL\text{-}X_L$), pro-proliferative (survivin, cyclin D1/D2), and pro-angiogenic proteins (MMP2, MMP9, and HIF1α). This persistent STAT3 activation also contributes to the increased production of ROS by MDSCs. A selective inhibitor of the Jak2/STAT3 pathway, JSI-124 (curcubitacin I), was capable of increasing immune responses against tumors (Blaskovich, Sun et al. 2003). This molecule markedly reduced the number of $CD11b^+/Gr\text{-}1^+$ immature myeloid cells in the tumor microenvironment, both by increasing their apoptosis (up to 50% in a colon carcinoma model) and by promoting their differentiation into more mature cells. Another category of drugs comprises molecules already used in the clinic, which might target STAT3 in addition to other factors. Sunitinib, a tyrosine kinase inhibitor, is for example used for the treatment of several tumor types for its alleged anti-angiogenic properties (Ko, Zea et al. 2009, Ugel, Delpozzo et al. 2009). Sunitinib also acts by inhibiting the STAT3 pathway in renal carcinoma-associated MDSCs. Antiangiogenic therapy based on the modulation of VEGF has also been considered as a possible approach to manipulate MDSC expansion. VEGF is a key regulator of physiological angiogenesis during embryogenesis,

skeletal growth, and reproductive functions, and it has been implicated in pathological angiogenesis associated with tumor development (Ferrara, Gerber et al. 2003). VEGF also affects bone marrow-derived cells, promoting monocyte chemotaxis and inducing colony formation by subsets of granulocyte-macrophage progenitor cells. VEGF-A blockade using the humanized anti-VEGF-A mAb bevacizumab (Avastin) was found to inhibit significantly angiogenesis and growth of human tumor xenografts (Shojaei and Ferrara 2008). Avastin was the first antiangiogenic agent approved by the Food and Drug Administration of this antibody was shown to cause also a decrease in the pool of a MDSC subset ($CD11b^{+}VEGFR1^{+}$ cells) in the peripheral blood of RCC patients (Kusmartsev, Eruslanov et al. 2008). Another possible target for a therapeutic approach to block MDSC expansion is the matrix metalloproteinase-9 (MMP-9). MMPs are a family of closely related, zinc-dependent proteolytic enzymes, that are able to degrade all the components of the extracellular matrix and, therefore, they are involved in a great number of physiological and pathological processes. MMP9 regulated the mobilization of hematopoietic stem cells from the bone marrow niche and MDSC expansion by solubilizing the membrane form of c-Kit (Heissig, Hattori et al. 2002) and making VEGF available. MMP-inhibitors can block the MMP-9 expression at the tumor site, halting this loop and normalizing hematopoiesis, thus reducing both myeloid support to tumor stroma and MDSC generation (Melani, Sangaletti et al. 2007). Amino-biphosphonates (BPs) represent an interesting alternative since they have shown excellent results in terms of safety and tolerance. These molecules interfere with protein prenylation and with the conversion of mevalonate to geranylgeranylpyrophosphate necessary for cholesterol synthesis. The BP treatment reduced both myelopoiesis and tumor stroma formation, enhancing tumor necrosis. In fact, this pharmacological treatment significantly reduced both the tumor stroma and the infiltration of inflammatory $CD11b^{+}Gr\text{-}1^{+}F4/80^{+}$ cells (Drake, Clarke et al. 2008). These studies also confirmed that a prolonged treatment with BP had no detectable toxicity in mice. In cancer patients a single dose of pamidronate and zoledronate decreased serum levels of VEGF, TGF-β and MMP-2 (Rogers T., 2011, J transl med) Another potential target to limit MDSC accumulation are the family of S100

calcium-binding protein A8 and A9 (S100A8/S100A9) together with their receptor for advanced glycation end-products (RAGE), (Cheng, Corzo et al. 2008). Accordingly, the injection of, an anti-carboxylate glycan antibody (mAbGB3.1) that blocks S100A8/A9 binding and signaling, reduced MDSC accumulation in blood and secondary lymphoid organs of tumor-bearing mice (Sinha, Okoro et al. 2008). The inhibition of colony stimulating factor 1 receptor (CSF-1R) signaling through small-molecule CSF1R kinase inhibitors constitutes another strategy to affect MDSC development; as an example, GW2580 abrogated tumor recruitment of $CD11b^{+}Gr\text{-}1^{lo}Ly6C^{hi}$ MO-MDSCs in mice bearing Lewis Lung Carcinoma (LLC) and also induced a decrease in expression of proangiogenic and immunosuppressive genes (Priceman, Sung et al. 2010). Today, a monoclonal antibody against CSF-1R (IMC-CS4) is undergoing a phase I clinical trial (NCT01346358) to establish its safety and pharmacokinetic profile in the treatment of subjects with advanced solid tumors, either refractory to standard therapy or for whom no standard therapy is available (Swierczak, Cook et al. 2014). Finally, a multitargeted tyrosine kinase inhibitor (PLX-3397, Pexidartinib) able to target also the CSF-1R pathway is tested in ongoing phase I/II clinical trials both in solid and hematological tumors (Ries, Cannarile et al. 2014).

Targeting MDSC differentiation. In addition to targeting suppressive functions, myelopoeisis can be diverted away from generating MDSCs for therapeutic benefit. All-trans-retinoic acid (ATRA), supplemented with GM-CSF, has been shown to differentiate MDSCs into DCs and improve their immune-stimulatory capacity, inducing the up-regulation of HLA-DR, CD1a and CD40 on MDSCs (Kusmartsev and Gabrilovich 2006, Kusmartsev, Eruslanov et al. 2008). Treatment of renal cell carcinoma patients with ATRA substantially decreased the presence of MDSCs in peripheral blood (Mirza, Fishman et al. 2006). A recent study demonstrated that lung cancer patients vaccinated against p53 showed a better immune response when immunotherapy was combined with the administration of ATRA (Iclozan, Antonia et al. 2013). Similar to ATRA, vitamins could also act as differentiating agents, as observed in two cohorts of newly diagnosed HNSCC patients who did or did not receive

$1,25(OH)_2D_3$ vitamin treatment for 3 weeks before surgical resection. Only treated patients showed a reduction in intratumor $CD34^+$ and immature DCs, paralleled by increased amounts of intratumor mature DCs (Kulbersh, Day et al. 2009). Another study in NSCLN patients treated with $1,25(OH)_2D_3$ revealed a discordant relationship between systemic and intratumoral cytokine profiles. Plasma Th1 cytokine levels in HNSCC patients were comparable to healthy controls while HNSCC tissues showed higher Th1 cytokine amounts than normal tissue. The treatment with $1,25(OH)_2D_3$ resulted in a trend towards an overall stimulation, not only for both Th1 and Th2 cytokines in plasma, and in tissue, but also for circulating angiogenic and pro-tumorigenic cytokines (Walker, Reeves et al. 2012) (Table1).

Drug	Type(s) of cancer	Effect(s) on myeloid cells
5-Fluorouracil	Thymoma	MDSC apoptosis
Gemcitabine	Sarcoma, lung, and breast cancer	MDSC apoptosis
Doxorubicin-cyclophosphamide	Breast cancer	MDSC apoptosis
Docetaxel	Mammary carcinoma	MDSC apoptosis
Gr-1-specific antibody	Colon carcinoma	MDSC depletion
Ly6G-specific antibody	PDAC	MDSC depletion
CD124 (IL-4Rα) targeting with aptamers	Mammary cancer	MDSC and TAM depletion
CCL2-specific antibody	Mammary carcinoma	MDSC recruitment and angiogenesis
CXCR2 and CXCR4 antagonists	Breast cancer	MDSC recruitment
AT38	Fibrosarcoma and thymoma	Impairment of MDSC suppression and reduced recruitment
PROK2-specific antibody	Various mouse and human tumors	Reduced MDSC expansion and recruitment
Nitroaspirin	Colon carcinoma	Inhibition of MDSC-dependent immune suppression
Triterpenoids	Thymoma, colon, and lung carcinoma	Inhibition of MDSC-dependent immune suppression
Tyrosine kinase inhibitor (sunitinib)	Human RCC, fibrosarcoma, and colon, breast, lung, and kidney tumors	Low inhibition of MDSC expansion in patients
Cyclooxygenase-2 inhibitors	Mammary carcinoma, mesothelioma, lung carcinoma, and glioma	Inhibition of MDSC-dependent immune suppression
PDE5 inhibitors (sildenafil, tadalafil)	Breast and colon cancer, human myeloma, and HNSCC	Inhibition of MDSC-dependent immune suppression
ATRA	Sarcoma, colon carcinoma, and human RCC	MDSC differentiation in mature cells
1α25-Dihydroxyvitamin D_3	HNSCC	MDSC differentiation in mature cells
CSF-1R antagonist	Prostate tumor and lung carcinoma	Reduced MDSC expansion and recruitment
CSF-1R monoclonal antibody (RG7155)	MC38 colon carcinoma and diffuse-type giant cell tumor	TAM depletion
GM-CSF-neutralizing antibody	Pancreatic cancer	Inhibition of proliferation
G-CSF-neutralizing antibody	Colon carcinoma	Inhibition of proliferation
VEGF-A-specific antibody (bevacizumab)	RCC	Inhibition of proliferation
IL-6R-specific antibody and gemcitabine	Methylcholanthrene-derived carcinoma cells	Reduced MDSC recruitment
Bisphosphonates	Mammary tumor	TAM depletion, inhibition of MDSC expansion
Combined therapy with IL-12, IL-16, CpG DNA, and IL-10R-specific antibody	Lung and breast cancer	TAM reprogramming
CD40 agonist and gemcitabine	PDAC	TAM reprogramming
CD40 antibody with IL-2	RCC	TAM reprogramming in lung metastasis but not in primary tumor
HRG	Pancreatic and breast cancer, fibrosarcoma	TAM reprogramming

Table1. A synopsis of drugs targeting MDSCs
Adapted from De Sanctis F., et al., 2016

2 Two converging pathways of apoptosis initiation

Apoptotic cell death is essential for normal development and tissue homeostasis. By activating the caspase family proteins, apoptosis triggers the activation of a proteolytic cascade that drives subsequent biochemical events leading to cell death. Deregulated apoptosis is an hallmark of several diseases, including autoimmunity, neurodegeneration and cancer (Dickens, Powley et al. 2012). Two major interplaying apoptotic signaling pathways, the intrinsic mitochondrion-initiated pathway and the extrinsic or cell surface death receptor pathway, are involved in apoptosis. In the mitochondrial pathway, caspase activation is closely linked to the mitochondrial outer membrane permeabilization (MOMP) by multiple distinct cytotoxic stimuli and pro-apoptotic members of the Bcl family. In particular, MOMP is regulated by proteins from the Bcl-2 family, mitochondrial lipids, proteins that regulate the bioenergetic metabolite flux and components of the permeability transition pore (Green and Kroemer 2004). Upon disruption of the outer mitochondrial membrane, a set of proteins of the intermembrane space are released, including cytochrome c, Smac/DIABLO (second mitochondria-derived activator of caspases), Omi/HtrA2 (a mitochondrial serine-protease), AIF (apoptosis inducing factor) and endonuclease G. Once in the cytosol, these apoptogenic proteins trigger the execution of cell death by promoting caspase activation or by acting as caspase-independent death effectors (Saelens, Festjens et al. 2004). The release of cytochrome c from mitochondria directly triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex, which becomes a platform on which the initiator caspase-9 is recruited and activates the executioner caspase-3 (Cain, Bratton et al. 2000, Fulda and Debatin 2006). By contrast, the induction of the extrinsic death pathway involves transduction of the pro-apoptotic signal after the binding of death receptors, such as CD95 (APO-1/Fas), TNF receptor 1 (TNFR1) or TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2, to their corresponding ligands FasL, TNF and TRAIL. Stimulation of these receptors by their cognate ligands or agonistic antibodies results in receptor trimerization and triggers the formation of a multiprotein death-inducing signaling complex

(DISC) (Kischkel, Hellbardt et al. 1995), which comprises the receptors, the adaptor molecule Fas-associated death domain protein (FADD) and the initiator caspases 8 and 10. In both apoptotic pathways, the activation of the initiator caspases results in the cleavage of the effector pro-caspase-3 that, in turn, is responsible for the proteolytic cleavage of a broad spectrum of cellular targets, leading ultimately to cell death. The known cellular substrates include structural components (such as actin and nuclear laminin), regulatory proteins (such as DNA-dependent protein kinase), inhibitors of deoxyri-bonuclease (such as DFF45 or ICAD), and other apoptotic proteins and caspases (Savitskaya and Onishchenko 2015).

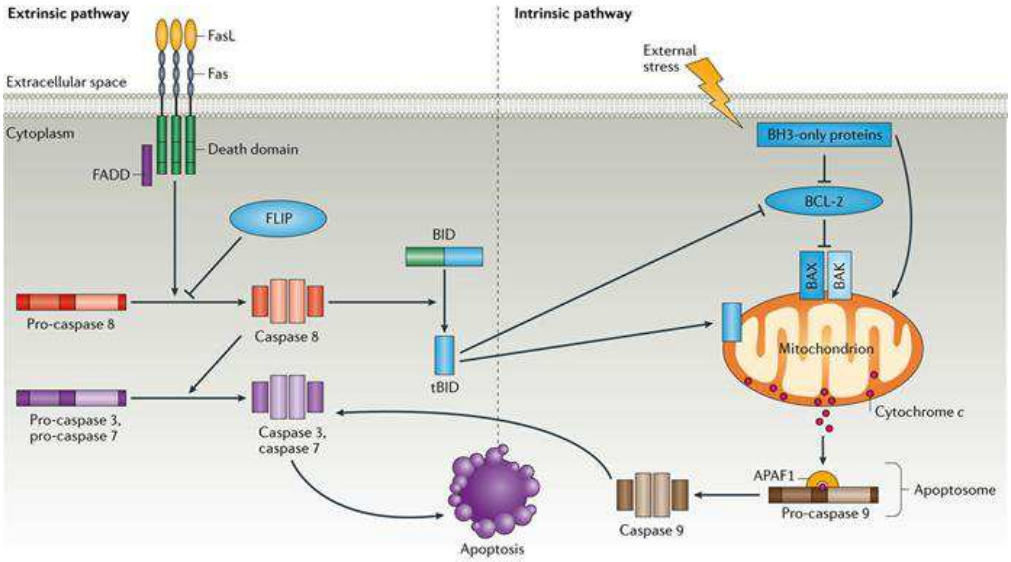

Figure 4. Extrinsic and intrinsic pathways of apoptosis. In an example of the extrinsic pathway of apoptosis, the binding of Fas ligand (FasL) to its receptor Fas leads to the cleavage and activation of caspase 8. This proteolytic step is mediated by the adaptor protein Fas-associated death domain protein (FADD) and can be inhibited by FADD-like apoptosis regulator (also known as FLIP), a catalytically inactive homologue of caspase 8. Subsequently, caspase 8 cleaves and activates caspase 3 and caspase 7, which in turn induce the degradative phase of apoptosis. In the intrinsic pathway of apoptosis, the BCL-2 homology region 3 (BH3)-only proteins can either sequester anti-apoptotic proteins such as BCL-2, or directly activate the pro-apoptotic multi-BH3 proteins, such as BAK and BAX. Once the apoptotic signaling is initiated, BAK and BAX induce the release of cytochrome c from the mitochondrion. Subsequently, cytochrome c binds to APAF1 and forms a complex with pro-caspase 9 (apoptosome). Activation of caspase 9 in the apoptosome in turn induces apoptosis through the activation of caspase 3 and caspase 7. An alternative pathway of Fas-induced cell death involves crosstalk between the extrinsic and the intrinsic apoptotic pathways. This crosstalk is mediated by the truncated form of BID (tBID), which induces apoptosis by translocating to the mitochondrion. *Adapted from Cuda et al., 2016*

2.1. Regulation of apoptosis.

The intrinsic apoptotic death pathway and, in particular, the integrity of the mitochondrial membrane is regulated by proteins of the Bcl-2 family. This protein family, which comprises both anti-apoptotic and pro-apoptotic proteins, is divided into three groups based on their domain architecture. The first group consists of anti-apoptotic members like Bcl-2, Bcl-XL, Bcl-w, Mcl-1, Bfl-1/A1, which are associated with the mitochondrial outer membrane, serving to maintain mitochondrial integrity. The second group is of the so-called BH3-only proteins, which act as sentinels over various cellular organelles and processes. Under normal conditions, the BH3-only proteins are inactive but, upon activation, they become activators of the third group of Bcl-2 family members, the large pro-apoptotic members Bax, Bak and Bok. These proteins associate with the outer mitochondrial membrane during apoptosis, breaching its integrity and causing the release of the inner mitochondrial proteins (Chawla-Sarkar, Bae et al. 2004). Another very relevant family of apoptosis regulators is composed by the inhibitors of apoptosis proteins (IAP) that are involved in cell death, immunity, inflammation, cell cycle and migration. Among the 8 members of this family, all characterized by the presence of one to three baculoviral IAP repeats (BIR) domains, c-IAP1 (BIRC2), c-IAP2 (BIRC3), ML-IAP (BIRC7 or livin) and XIAP (BIRC4) are directly involved in apoptosis regulation. c-IAP1 and c-IAP2 regulate the extrinsic apoptotic pathway through their ubiquitin ligase activity. In fact, c-IAP proteins are responsible for RIP1 ubiquitination: in their absence RIP1 cannot be ubiquitinated. Non-ubiquitinated RIP1 can form a cytosolic complex with the adaptor molecule FADD and caspases-8, leading to induction of apoptosis. Moreover, XIAP proteins can inhibit apoptosis by binding with strong affinity and inactivating the initiator caspase-9 and the effector caspases-3 and -7. XIAP inhibitory function can be blocked by SMAC that in turn can be inhibited by ML-IAP (de Almagro and Vucic 2012, Kaufmann, Strasser et al. 2012). Finally, another major regulator of the extrinsic apoptotic pathway is cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP), a

critical anti-apoptotic and drug resistance factor that negatively regulates the signaling complex downstream the death receptors.

3. Cellular FLICE-like Inhibitory Protein (c-FLIP).

The first observation of a new family of anti-apoptotic proteins was made in 1997 by Thome et al. who showed that cells expressing high level of viral FLICE-inhibitory proteins (vFLIPs) were resistant to Fas, TRAILR1 and TNFR1-induced apoptosis (Thome, Schneider et al. 1997). vFLIP proteins are components of γ-herpesvirus such us equine herpesvirus-2 (EHV-2), herpesvirus saimiri (HVS), the Kaposi-associated human herpesvirus-8 (HHV-8), the rhesus rhadinovirus (RRV) and the human molluscipoxvirus (MCV), which contains two additional v-FLIP variants with carboxy-terminal extensions of unknown function (Bertin, Armstrong et al. 1997, Hu, Vincenz et al. 1997). In mammalians, the cellular homologue is named c-FLIP (also called FLAME-1, I-FLICE, Casper, CASH, MRIT, CLARP and usurpin) and the gene is evolutionarily well conserved in mouse and human. About 13 distinct spliced variants have been characterized, three of which are expressed as proteins: the 26 kDa short form (c-$FLIP_S$), the 24 kDa form (c-$FLIP_R$, specifically expressed in some cell lines such as Raji and SKW6.4 and in human primary T cells) and the 55 kDa long form (c-$FLIP_L$) (Golks, Brenner et al. 2005, Bagnoli, Canevari et al. 2010). In humans, the decision to make c-$FLIP_S$ or c-$FLIP_R$ is defined by a single nucleotide polymorphism in a 3´ splice site of the c-FLIP gene. An intact splice site directs production of c-$FLIP_S$, but the splice-dead variant causes production of c-$FLIP_R$ (Ueffing, Singh et al. 2009). The structures of c-$FLIP_S$ is similar to v-FLIP proteins with two death effector domains (DEDs) that are peculiar of this family of proteins. c-$FLIP_R$ also contains two DEDs but lacks the additional carboxy (C)-terminal amino acids that are present in c-$FLIP_S$. The C-terminus of c-$FLIP_L$ is longer than that of c-$FLIP_S$ and closely resembles the structure of caspases-8 and -10, but catalytically inactive. This lack of caspase activity is the result of several amino acid substitutions, particularly the crucial cysteine residue in the catalytic

domain that is necessary for the catalytic activity of caspases. Additionally, c-$FLIP_L$ has a caspase-8 cleavage site at position Asp-376 (LEVD); c-$FLIP_L$ cleavage at this site produces the proteolytic fragment variant p43. The C-terminal region of c-$FLIP_S$ and c-$FLIP_R$ play a crucial role in ubiquitination and degradation, as well as the anti-apoptotic function of these isoforms (Lavrik and Krammer 2012, Safa 2013).

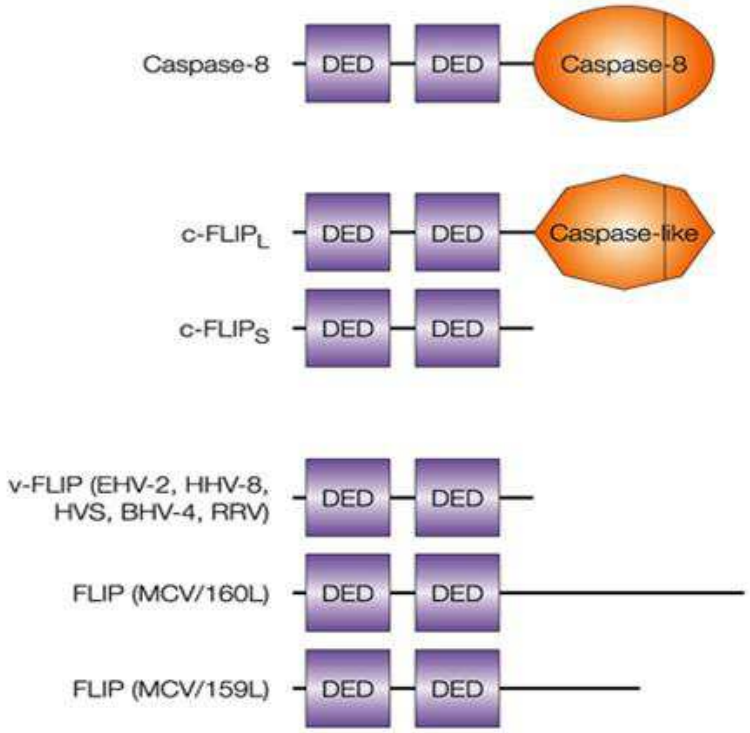

Figure 5. Molecular structure of viral and cellular FLIPs. Both herpesviral FLIP and short form of c-FLIP consist essentially of two repeats of a death effector domain (DED). The long splice variant of c-FLIP contains a carboxy- terminal inactive caspase-like domain,which confers on the molecule an overall structural homology with caspase-8 and caspase-10.
Adapted from Thome M., et al., 2001

c-FLIP is present in many tissues, most abundantly in the heart, skeletal muscle, lymphoid tissues and kidney. c-FLIP expression is very well regulated both at the transcriptional, translational and post-translational levels by several stimuli: the

transcription of c-FLIP gene can be activated by TNF ligands, growth factors, interleukins, chemokines ad chemotherapeutic agents (Safa, Day et al. 2008) and several mediators have been identified. These include NF-κB, p63, nuclear factor of activated T cells (NFAT), heterogeneous nuclear ribonucleoprotein K (hnRNPk), Early growth response-1(EGR1), androgen receptor (AR) and SP1 that are known to induce c-FLIP expression and, on the contrary, c-myc, the forkhead transcription factor Foxo3a, c-Fos, IRF5, and SP3 that suppress c-FLIP transcription. p53 may transcriptionally upregulate the c-FLIP gene and also promote the degradation of c-FLIP protein. In addition, c-FLIP seems to play a key role in the NF-κB-mediated control of death signals. c-FLIP binds TRAF2 and RIP (mediators of the anti-apoptotic signals from TNF receptors), resulting in strong NF-κB activation upon stimulation of death receptors. Thus, c-FLIP expression levels are augmented by an auto-amplification loop, determining whether a cell is resistant or sensitive to apoptosis (Micheau, Lens et al. 2001). In particular, it was recently demonstrated that c-FLIP isoforms do not directly compete with procaspase-8 for binding to FADD but c-FLIP recruitment to the DISC is a cooperative and hierarchical process in which procaspase-8 binding to FADD is the primary initiating event, which in turn promotes recruitment of c-FLIP via heterodimerization with procaspase-8. c-$FLIP_L$ can both activate or inhibit procaspase-8, highlighting that the ratio of c-$FLIP_L$ to procaspase-8 is critical in determining the fate of the cells: high concentration of c-$FLIP_L$, relative to caspase-8, mediates the inhibition; on the contrary, physiological concentration of c-$FLIP_L$ preferentially activates the procaspase-8. c-$FLIP_s$ is a potent inhibitor of apoptosis completely blocking procaspase-8 processing, disrupting DED-mediated procaspase-8 oligomer assembly and preventing the cleavage of caspase-8. The balance of c-$FLIP_{L/S}$ to procaspase-8 is critical in determining signaling outcome, and a shift in the concentration or stoichiometry of these molecules determines signaling for death or survival (Hughes, Powley et al. 2016).

3.1. c-FLIP in immune cells.

c-FLIP isoforms have a key role in the homeostasis of many population of immune cells. In B cells, c-FLIP is part of the regulatory mechanism that determines survival of germinal center B cells after successful affinity maturation of the BCR (Hennino, Berard et al. 2001). c-FLIP, with the concerted action of CD40 and FAS, renders the autoreactive B cells sensitive to Fas apoptosis and can mediate the elimination of B cells after the initial activation and proliferation, preventing abnormal B-cell expansion, production of autoantibodies and development of autoimmune diseases (Siegel, Chan et al. 2000). c-FLIP plays also a relevant role in clonal deletion of T cells; c-FLIP expression has been found to be first upregulated and then downregulated in primary T cells after antigenic stimulation and T cells activation (Thome, Schneider et al. 1997). The downregulation of c-FLIP is mediated by IL-2; in fact, it is not observed in IL-2 knockout mice (Refaeli, Van Parijs et al. 1998). Also macrophages, which differentiate from circulating blood monocytes and switch from a FAS-sensitive to a FAS-resistant phenotype, and DCs during their differentiation process into mature cells increase the expression of c-FLIP (Perlman, Pagliari et al. 1999, Huang, Perlman et al. 2010). In human, the activation of monocytes results in selective resistance to apoptosis particularly to signaling via death receptors and DNA damage. Rapid induction of the anti-apoptotic member and a marked reduction in the expression of the most apical caspase, i.e., caspase-8 provide a potential mechanistic basis for the activation-induced resistance to apoptosis in human monocytes (Perera and Waldmann 1998). Finally, it has been recently demonstrated that in mouse, c-FLIP is constitutively required for the development and survival of M-MDSCs, thus preventing caspase-8-dependent apoptosis. Indeed, mice lacking c-FLIP in the late myeloid lineage have an hyperproduction of granulocytes and a relative depletion of monocytes (Haverkamp, Smith et al. 2014).

3.2. c-FLIP under pathological context.

Tumor cells can evade apoptosis through several mechanisms that affect both intrinsic and extrinsic apoptosis pathways. c-FLIP has been found to be overexpressed in several types of malignancies and could be associated with cancer progression due to its ability to inhibit the apoptotic process. Overexpression of c-FLIP is associated with an increased resistance to apoptosis mediated by Fas and TRAIL, and studies have demonstrated that in some tissue types, high levels of c-FLIP expression correlates with a more aggressive tumor (Djerbi, Screpanti et al. 1999). Overexpression of c-FLIP has been reported in colorectal cancer (Ullenhag, Mukherjee et al. 2007), bladder urothelial cancer (Korkolopoulou, Goudopoulou et al. 2004), cervical cancer (Wang, Wang et al. 2007), Burkitt's lymphoma (Valnet-Rabier, Challier et al. 2005), non-Hodgkin's lymphoma (Valente, Manfroi et al. 2006), Head and Neck Squamous Cell Carcinoma (HNSCC) (Li, Pan et al. 2008), hepatocellular cancers (Du, Bao et al. 2009), and Prostate Cancer (CRPC) (McCourt, Maxwell et al. 2012). c-FLIP can also be responsible for resistance to multiple anticancer drugs (Mathas, Lietz et al. 2004, Galligan, Longley et al. 2005, Longley, Wilson et al. 2006, Thorburn, Behbakht et al. 2008) highlighting c-FLIP as critical target for therapeutic intervention. Small molecules that selectively downregulate c-FLIP$_S$ or c-FLIP$_L$ and gene therapy strategies that knock down a specific c-FLIP variant have been used to downregulate these isoforms. c-FLIP isoforms can be inhibited by compounds that inhibit their transcription or translation, trigger their degradation, or by c-FLIP-specific small interfering RNA (siRNA), which sensitize a wide range of cancer cell types to apoptosis. Pretreatment with DNA damaging drugs such as cisplatin, 5-FU, oxaliplatin, doxorubicin and camptothecin is effective in inducing the downregulation of c-FLIP isoforms in various tumor cells by inhibiting its transcription and rendering cells sensitive to death receptor-triggered apoptosis (Kinoshita, Yoshikawa et al. 2000, Longley, Wilson et al. 2006, Abedini, Muller et al. 2008). In addition, histone deacetylase inhibitor (HDACi) compounds have been shown to downregulate c-FLIP expression in various cancer cells both at the transcriptional and translational levels (Wood, Dalili et al.

2010, Yerbes and Lopez-Rivas 2012) and a number of agents that modulate Akt, PI3K, NF-κB, and Ras pathways, as well as an inhibitor of STAT3, have also been shown to transcriptionally silence c-FLIP expression (Li, Zhang et al. 2009, Jani, DeVecchio et al. 2010, Liu, Wang et al. 2010).

AIM OF THE STUDY

MDSCs are a heterogeneous population of cells at different maturation stages that have the potential activity to suppress immune responses of T lymphocytes, both *in vitro* and *in vivo,* creating a microenvironment capable of altering the immune response (Bronte, Brandau et al. 2016). The most interesting MDSC subset is represented by the M-MDSCs, mainly comprising monocytes and myeloid precursors, capable to suppress strongly $CD8^+$ T-cell activation, both *in vitro and in vivo*. To mimic MDSC differentiation, we took advantage of an *in vitro* culture to generate MDSCs based on the short term incubation of whole BM cells with a cytokine combination of GM-CSF and IL-6 (Marigo, Bosio et al. 2010). Using this useful strategy, we tested the immune- properties of first-generation chemotherapeutics on modulating MDSC differentiation and we identified a common mechanisms through which these drugs exert their action. Recent data demonstrated that mouse MDSC heterogeneity between the two main subsets, the M-MDSC and the PMN-MDSCs, occurs from a diverse activation of molecular pathways regulating the death-inducing signaling complex and, in particular, the development, survival and function of M-MDSCs constitutively require the presence of c-FLIP (Haverkamp, Smith et al. 2014). Therefore, we verified the hypothesis that the common chemotherapy-induced effect on M-MDSC contraction was mediated by the down-regulation of c-FLIP expression.

The second aim of this work was the demonstration of the potential role of c-FLIP to drive and control the M-MDSC-related immunosuppressive function in order to unveil a novel biomarker able to link the immunomodulatory properties of these cells and predict the response to therapy in cancer patients. For this aim, we used lentiviral expressing vectors encoding the two major splice variants of human c-FLIP to up-upregulate the expression of the target gene on human $CD34^+$ cells, during their differentiation into human MDSCs, and human $CD14^+$ monocytes, and demonstrate that c-FLIP controlled the immune regulatory program. Moreover, the therapeutic potential of human c-FLIP-infected monocytes was assessed *in vivo* using a xenogeneic mouse model of GvHD.

Finally, we validated the hypothesis about c-FLIP as a master regulator of immunosuppression, using a transgenic mouse model expressing the viral form of c-FLIP (vFLIP) in the myeloid lineage: the Rosa26.vFLIP knock-in mice bred with mice expressing the Cre recombinase under the control of the endogenous Lyz2 promoter. After a characterization of this mouse model we verified the suppressive activity of $Ly6C^+$ cells isolated from Tg mice.

All these data confirm the central role of c-FLIP on the activation of MDSCs-related immunosuppressive properties.

MATERIAL and METHODS

1. Mice

NOD.Cg-*Prkdcscid Il2rgtm1Sug*/JicTac (NOG) mice were purchased from Taconic Biosciences. C57BL/6 (H-2b) mice were purchased from Charles River Laboratories Inc. OT-1 TCR-transgenic mice (C57Bl/6-Tg(TcraTcrb)1100Mjb/J) and CD45.1^{+} congenic mice (B6.SJL-Ptrca Pepcb /BoyJ) were from Jackson Laboratories. Rosa26.vFLIP mice were a gift from Dr. Cesarman. All mice were maintained under specific pathogen-free conditions in the animal facilities of the University of Verona. Animal experiments were performed according to national (protocol number 12722 approved by the Ministerial Decree Number 14/2012-B of January 18, 2012) and European laws and regulations. All animal experiments were approved by Verona University Ethical Committee (http://www.medicina.univr.it/fol/main?ent=bibliocr&id=85) and conducted according the guidelines formulated by the council of Europe for experimental animal use. All animal experiments were in accordance with the Amsterdam Protocol on animal protection and welfare. Additionally, all animal experiments were conducted according to the guidelines of Federation of European Laboratory Animal Science Associations (FELASA).

2. Cell lines.

Chicken ovalbumin (OVA)-transfected EL-4 (EG7) and Human embryonic kidney cell line 293 (HEK293) were grown in DMEM (Invitrogen) supplemented with 2 mM L-glutamine, 10 mM HEPES, 20 mM b-mercaptoethanol, 150 U/ml streptomycin, 200 U/ml penicillin, and 10% heat-inactivated FBS (Invitrogen). MSC-2 is an immortalized cell line and were cultured in RPMI 1640 (Life Technoloies) with 10% fetal bovine serum enriched with 0.4mmol/L of sodium pyruvate, 4mmol/L of HEPES and 200 U/ml penicillin.

3. Cytokines and peptides.

Mouse recombinant GM-CSF and mouse recombinant IL-6 were purchased fromMiltenyi Biotec. Kb -restricted OVA257-264 peptide (SIINFEKL), was

synthesized by JPT (Berlin, Germany). Necrostatin-1 was purchased from Sigma-Aldrich and Pan Caspase Inhibitor Z-VAD-FMK from R&D Systems. Human recombinant GM-CSF and human recombinant G-CSF was purchased from Sanofi Aventis.

4. Mouse proliferation assay.

The immunosuppressive activity was evaluated plating the *in vitro* differentiated MDSC or the freshly isolated myeloid cells from ROSA26.vFLIP mice in 96 wells plate at a final concentration of 24% of total cells in culture in presence of splenocytes from OT-I transgenic mice, labeled with 1 μM CellTrace and diluted 1:10 with $CD45.1^+$ splenocytes, in the presence of SIINFEKL peptide (1 μg/ml final concentration). After 3 days of co-culture, cells were stained with APC-Cy7 conjugated anti-CD45.2 (104, eBioscence) and PerCP-Cy5.5 conjugated anti-CD8 (SK1, eBioscience), and CellTrace signal of gated lymphocytes was analyzed. we performed FACS analysis with a FACS-Canto (BD Biosciences, NJ, U.S.A.) and, to evaluate the suppression of proliferation, plots were gated on CD8 + T cells and the percentage of cells that had diluted Cell trace was evaluated using FlowJo software (Treestar Inc.). The percentage of proliferating cells was then used to calculate the percent suppression of proliferation as described below. Percent suppression of proliferation was calculated using the following formula: 1 (% proliferation with MDSCs/% proliferation without MDSCs) × 100.

5. Human proliferation assay.

PBMCs were isolated from leukocyte-enriched buffy coats from healthy volunteer (Transfusion Center) by Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) gradient centrifugation as described above. PBMCs were then counted, frozen at -80°C and stored in liquid nitrogen. To analyze cell proliferation, an *in vitro* labelling system was used to trace multiple cell divisions using dye dilution by flow cytometry. PBMCs were recovered and washed in IMDM medium (Lonza, Switzerland), supplemented with 10% FBS (EuroClone, Milan, Italy), 100U/ml penicillin/streptomycin (Lonza, Switzerland), β-mercaptoethanol (Sigma-Aldrich, Milan, Italy) and 10 mM HEPES (Lonza, Switzerland). PBMCs were resuspended at a final concentration of 10^7 cells/ml in PBS and stained with 2,5 μM as final

working concentration of CellTrace Violet stock solution (Invitrogen Molecular Probe), followed by 5 minutes incubation at 37°C, protected from light. Cells were then washed and resuspended in culture medium. Labelled "target" PBMCs were stimulated with coated 0.6 μg/ml anti-CD3 (clone OKT-3) and 5 μg/ml soluble anti-CD28 (clone CD28.2, eBioscience) for four days and co-cultured with "effectors" $CD34^+$ or $CD14^+$ cells at 0.1:1, 0.5:1, 1:1, 2:1, 3:1 ratios (effector : target) in 384 flat bottom well plates (BD Biosciences, NJ, USA). Cell cultures were incubated at 37°C and 5% CO_2 in arginine and glutamine–Free-RPMI (Biochrom AG, Berlin, Germany), supplemented with 2 mM L-glutamine, 150μM arginine, 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 10 U/ml penicillin and streptomycin (Lonza, Switzerland), and 0.1 mM HEPES (Lonza, Switzerland). At the end of the culture, cells were stained with PE-Cy7 conjugated anti-CD3 (UCHT1, eBioscience), and CellTrace signal of gated lymphocytes was analyzed. The extent of cell proliferation was quantified by FlowJo, analyzing the number of proliferating cells, assumed to be 100% without effector cells.

6. Plasmid vectors.

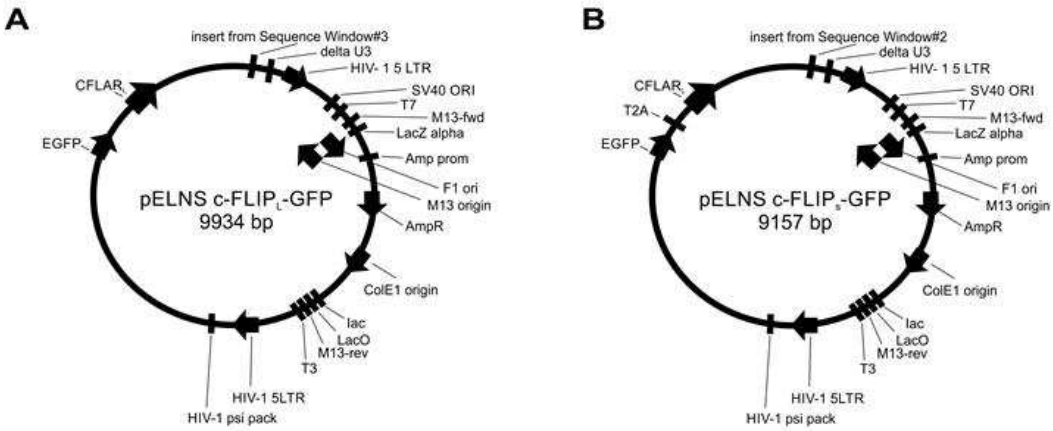

Figure 1. c-FLIP$_{long}$ (A) and c-FLIP$_{short}$ (B) plasmid vectors

7. Immunomagnetic $CD14^+$ cells isolation.

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat of healthy donors by density centrifugation. In brief, peripheral blood was diluted

1:2 with PBS and carefully layered onto a density gradient Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden). After centrifugation, the band of PBMCs was aspirated and washed three times with ice-cold PBS. PBMCs were then stratify into a density gradient Percoll-Media Hypaque (GE Healthcare, Uppsala, Sweden) in order to enrich the monocytes fraction. $CD14^+$ monocytes were then isolated with immunomagnetic sorting (Miltenyi, Bergisch Gladbach, Germany) according to manufacturer's instructions. Sorting purity was evaluated by flow cytometry using a mouse anti-human mAb.

8. Lentivirus Production and infection.

Self-inactivating replication of viral particles were produced by calcium phosphate-mediated transfection of packaging cells (HEK293T) with the appropriate amounts of vector transfer plasmid and HIV-1 lentiviral packaging constructs pDEL, pREV, and VSV-G. The virus supernatants were harvested 48 hours after transfection, 0.22 μm filtered, concentrated by ultracentrifugation (50,000 x g; 2,20 h), and titrated by limiting dilution assay in HEK293T.

Human $CD34^+$ cells were infected at a (multiplicity of infection) MOI of 2 with 8μg/ml of Polybrene (Millipore) and incubated in a 37°C incubator at 8% CO_2 overnight. The virus media was then removed and replaced with fresh media and cytokines. The cells were later on differentiated for four days. Human $CD14^+$ cells were infected at a MOI of 1 with 8μg/ml of Polybrene (Millipore) for four hours by spin-inoculation (2000rpm; 37°C). After, the virus media was diluted and cells were incubated in a 37°C incubator at 5% CO_2 overnight.

9. Real Time.

Total RNA from both c-FLIP or luciferase- infected monocytes was isolated by TRIzol reagent (Life technology, CA, U.S.A.) and RNA integrity assessed using Agilent-2100- Bioanalyzer (Agilent Technologies, CA, U.S.A.). The RNA was further purified with RNeasy MinElute Cleanup kit (Qiagen, Venlo, Netherlands) and cDNA was synthesized and amplified from total purified RNA with Ovation Pico WTA System V2 (NuGEN, CA, U.S.A.). All the samples were used to set up a Real Time PCR with custom primers designed by ABI Biosystems by Primer

Express. Post qRT-PCR analysis to quantify relative gene expression was performed by the comparative Ct method ($2^{-\Delta\Delta Ct}$).

10. Gene expression.

For transcriptome analysis, total RNA of c-FLIP- and luciferase-infected $CD14^+$ cells isolated from four healthy donor was isolated using TRIzol reagent (Life technology, CA, U.S.A.) and RNA integrity assessed using Agilent-2100-Bioanalyzer (Agilent Technologies, CA, U.S.A.). The RNA was further purified with RNeasy MinElute Cleanup kit (Qiagen, Venlo, Netherlands) and cDNA was synthesized.

All the samples were hybridized to Affymetrix U133 PLUS 2.0 arrays and scanned with an Affymetrix GCS 3000 7G scanner. Sample and genes are clustered using Pearson correlation coefficient and average as distance metric and linkage, respectively. Bioinformatic analysis was performed by Prof. Silvio Bicciato of University of Modena.

11. Elisa assays.

ELISA for human IL-10 (eBioscience, CA, USA) and ProcartaPlex Mouse Cytokine & Chemokine Panel 1A (36 plex) (eBioscience, CA, USA) were performed according to manufacturer's instructions.

12. Western blot.

Cell lysates were made in RIPA buffer with the addition of protease inhibitor cocktail tablets (Roche) and sodium vanadate. Insoluble material was removed by centrifugation. SDS-PAGE was done on 12% denaturing SDS polyacrylamide gel and transfered on PVDF membrane (Millipore). Membranes were blocked in Tris-buffered saline plus 0.05% Tween-20 and 5% non-fat milk.

We used the following antibodies: anti-mouse caspase-3 (D3E9), caspase-7 (D6H1), caspase-8 (D35G2) and caspase-9 (D2D4; Cell Signaling Technology; diluted 1:1,000). Following the hybridization with the secondary horseradish peroxidase (HRP)-conjugated, donkey anti-rabbit IgG Ab (Amersham Biosciences).

13. Flow cytometry.

Sample tubes were washed in phosphate-buffered saline (PBS) and incubated with FcReceptor Blocking reagent (Miltenyi Biotech) for 10 minutes at 4°C to saturate FcR. The following mAb were then used for cell labeling:

Antibody	Reactivity	Clone	Label	Company
CD11b	Mouse	M170	APC	eBioscience
Ly6C	Mouse	HK1.4	V450	eBioscience
Ly6G	Mouse	RB6-8C5	APC-Cy7	eBioscience
B220	Mouse	RA3-6B2	PE-Cy7	eBioscience
CD3	Mouse	145-2C11	PE	eBioscience
CD8	Mouse	SK1	PercP-Cy5.5	eBioscience
CD45.2	Mouse	104	APC-Cy7	eBioscience
CD34	Human	4H11	PE	eBioscience
CD33	Human	WM53	V450	BioLegend
CD14	Human	61D3	APC-Cy7	eBioscience
CD117	Human	104D2	PE-Cy7	BD Pharmigen
CD3	Human	UCHT1	PE-Cy7	eBioscience
CD274	Human	MIH1	V450	BD Pharmigen
CD38	Human	HIT2	APC	BD Pharmigen
CD80	Human	2D10	APC	BD Pharmigen
CD44	Human	IM7	PE	eBioscience
CD163	Human	GHI/61	APC	BD Pharmigen
CD273	Human	MIH18	APC	BD Pharmigen
CD124	Human	25463	PE	R&D Systems
CD4	Human	SK3	APC-Cy7	eBioscience
CD8	Human	RPA-T8	APC	BD Pharmigen
CD25	Human	BC96	PE	eBioscience
FoxP3	Human	259D/C7	FITC	BD Pharmigen
IFNγ	Human	B27	APC	BD Pharmigen

Samples were acquired with a FACSCanto II (BD Biosciences, NJ, USA) and analyzed with FlowJo software (Treestar Inc.).

14. Xenogenic model of GvHD.

NOG mice, were irradiated (120 rad) and intravenously (i.v.) injected with $1x10^6$ human peripheral blood mononuclear cells. After 21 days, when human $CD3^+$ cells where around 5% of total cells, mice were treated by intravenously injection

of c-FLIP- or Luciferase- infected monocytes. The treatment was repeated 4 times on day 21, 28, 42 and 49. Mice were monitored for immunological, clinical and histological scores.

15. Histology and Immunohistochemistry.

Tissues were removed at necroscopy and fixed in 10% buffered formalin and embedded in paraffin. Histopathologic evaluation was performed on standard hematoxylin-and-eosin sections. Pathological score was evaluated by two pathologists blinded using the standard line guide previously published (Cooke, Kobzik et al. 1996). Finally, paraffin sections of spleen, liver, lung, skin, colon, kidney and stomach have been used for the immunohistochemistry: sections were stained with anti-human CD3 (clone ab5690, Abcam, United Kingdom) antibody to quantify human tissues-infiltrating lymphocytes.

16. Statistical analysis.

All data are presented as mean ± standard deviation of the mean. Statistical analysis was carried out using SigmaPlot (Systat Software). For statistical comparison of two groups, non-parametric Mann-Whitney Wilcoxon test was used. A value of $P < 0.05$ was considered significant.

RESULTS

1. Chemotherapy susceptibility of mouse MDSCs.

Purified splenic $CD11b^{+}/Ly6G^{-}/Ly6C^{high}$ cells show morphological features of immature myeloid cells and comprise a population of inflammatory monocytes with high proliferative rate, multipotent progenitor features that, although partially committed, might contribute to myeloid replenishment in the spleen during tumor growth (Ugel, Peranzoni et al. 2012). Moreover, low doses of different chemotherapies can be used to selectively eliminate $CD11b^{+}/Ly6G^{-}/Ly6C^{high}$ cells, promoting a higher therapeutic effect in combination with adoptive cell therapy with tumor-specific T-cells. Interestingly, most of the drugs that alter the splenic immunoregulatory environment do not affect the immunogenic context of cancer death (Ugel, Peranzoni et al. 2012). Future developments could thus be based on optimizing low-dose chemotherapy to eliminate immunosuppressive monocytes before administering a specific drug that is able to elicit immunogenic death and increase tumor-antigen (Ag) uptake by APCs present in the tumor microenvironment. Starting from these observations, we tested several cytotoxic agents, used in conventional anti-cancer therapy, to verify their ability to modulate *in vitro* BM-MDSC vitality and differentiation. To mimic MDSC differentiation, we took advantage of a useful *in vitro* strategy to generate MDSCs based on the short-term incubation of whole BM cells with a cytokine cocktail of GM-CSF and IL-6 (Marigo, Bosio et al. 2010). We tested 12 drugs divided in 4 groups on the basis of their mechanism of action. The first family is constituted by the anti-metabolites like 5-fluorouracil (5-FU, active on thymidilate synthase), Gemcitabine (a deoxycytidine analogue that inhibits ribonucleotide reductase) and Fludarabine (purine analogue inhibiting DNA synthesis). The second class is represented by the mitotic inhibitors and comprises Vinblastine (a tubulin and microtubule poison), Docetaxel (able to prevent microtubule depolymerization), and Paclitaxel (interfering with the normal breakdown of microtubules during cell division). In the third group, the alkylating agents, we tested the platinum

compounds like Oxaliplatin (that prevents DNA replication forming inter and intra strand cross links in DNA), Carboplatin (that interferes with DNA repair mechanism), and Cisplatin (which promotes DNA adducts). The Topoisomerase inhibitors as Doxorubicin (that blocks Topoisomerase II), Etoposide (that causes DNA strands to break interfering with the Topoisomerase II) and Irinotecan (inhibitor of Topoisomerase I) represent the last class. For our analysis, we chose to evaluate the BM-MDSC differentiation change in a setting were >75% of cells were viable at the end of the culture (assessed by flow cytometer staining 7-AAD$^-$ /ANNEXIN-V$^-$). We tested a range of drug concentrations starting from a 90 µg/ml dose. These different chemotherapeutic drugs presented diverse toxicity; however, except for three drugs (Fludarabine, Carboplatin and Doxorubicin) which induced an high mortality (data not shown), all the tested treatments did not affect cell viability starting at 3 µg/ml dose. At this point, we proceeded by investigating the modulation on BM-MDSC differentiation in presence of the different chemotherapeutics. Interestingly, as previously observed in the *in vivo* experiments (Ugel, Peranzoni et al. 2012), the addition of the chemotherapy at the beginning of the culture, resulted in a dose-response redistribution of the myeloid subsets: indeed, flow cytometry analysis of the CD11b$^+$/Ly6G$^-$/Ly6C^{high} and CD11b$^+$/Ly6G$^+$/Ly6C$^{low/int}$ cell subsets, showed a significant contraction of the immunosuppressive monocytic cells in BM cultures. As shown in Figure 1A, B, C, within the indicatetd viability range, for all the tested drugs we identified both a "functional" dose (in red) able to selectively deplete the CD11b$^+$/Ly6G$^-$ /Ly6C^{high} (M-MDSCs) and leave intact, at the same time, the CD11b$^+$/Ly6G$^+$/Ly6C$^{low/int}$ cells (PMN-MDSCs) together with a "non-functional" one (in blue), which did not affect the two cell subsets. Furthermore, we evaluated the effect on the immune suppressive ability of the BM-MDSCs after the pharmacological treatment. As shown in Figure 1D and E, BM-MDSCs treated with the functional doses of chemotherapy (red bars) displayed a significant impaired suppressive activity compared to the untreated (UT) cells (in black) and the cells treated with non-functional doses (in blue). These data demonstrated that a specific chemotherapeutic dose, able to selectively contain the

expansion of M-MDSCs without affecting the PMN-MDSC cell population, is responsible for a full restoration of activated T cell proliferation.

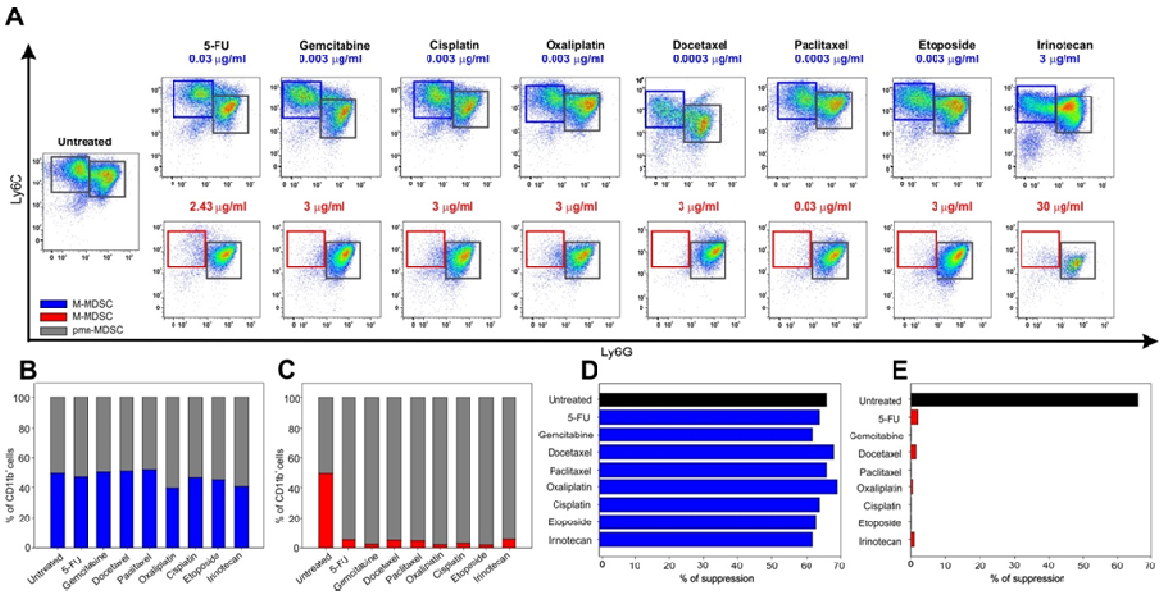

Figure 1. Chemotherapy affects MDSC differentiation and function. A) Representative flow cytometry plots of MDSC distribution after chemotherapy treatment with twelve different dugs, that, in a range of more than 75 % of viable cells ($AnnV^{-}/7AAD^{-}$), induce a contraction of M-MDSCs ($CD11b^{+}/Ly6C^{high}/Ly6G^{low}$), leaving unalterated PMN-MDSCs ($CD11b^{+}/Ly6C^{low}/Ly6G^{l}$). **B,C)** Graphs compare the percentage of M-MDSCs and PMN-MDSCs on the $CD11b^{+}$ fraction after the pharmacological treatments. In blue **(B)** the drug doses that don't affect the MDSC differentiation process, **(C)** in red the concentrations of drugs that affect the MDSC distribution. **D,E)** Suppressive activity of the untreated (in black) or chemotherapy treated MDSCs: the non-functional (in blue) or the functional (in red) doses of chemotherapy.

2. Activation of a common death pathway by different chemotherapeutics.

Trabectedin, a recently approved chemotherapeutic agent, was shown to induce a rapid and selective apoptosis in monocytes and macrophages by regulating signaling and decoy TNF-related apoptosis-inducing ligand (TRAIL) receptors (Germano, Tinessa et al. 2013) and the key effector molecule of this process was caspase-8. Based on these observations, we decided to investigate the potential activation of death pathways shared among the chemotherapeutics and responsible for $CD11b^{+}/Ly6G^{-}/Ly6C^{high}$ cell contraction during *in vitro* differentiation. We focused our attention to the extrinsic apoptotic pathway. For our study, we treated MSC2 cells, an immortalized myeloid suppressor cell line that well resemble BM-MDSCs (Apolloni, Bronte et al. 2000), with the functional doses of one pharmacological drug for each class that we previously tested, i.e. 5-FU, Cisplatin, Docetaxel and Irinotecan. We verified by western blot analysis the activation of the key caspases after 6, 12, 24 and 48 hours of pharmacological treatment. We specially investigated Caspase-8, Caspase-9, Caspase-7 (with antibodies able to recognize both the pro and cleaved caspases) and the cleaved Caspase-3. As shown in Figure 2A, although with different intensity, caspase cleavage was present in all the treated samples compared to untreated control. To validate further the activation of the extrinsic apoptotic death pathway and exclude the involvement of other pathways such as the necroptosis, we cultured the MSC2 cells for 24 hours in the presence of the functional dose of 5-FU alone or in combination with 50 µM pan-caspase Inhibitor (Z-VAD-FMK) or 50 µM necrostatin-1 (Nec-1), which targets RIPK1 activity and blocks the necrotic cell death. As shown in Figure 2B, we demonstrated that the addition of the pan-caspase inhibitor (green bar) is effective in restoring the cell viability affected by the treatment of 5-FU, whereas no rescue is detectable when cells are exposed to Nec-1 (in yellow). With the attempt to explain the common action of these different chemotherapeutic drugs, we verified whether the pharmacological treatment affected the expression level of c-FLIP on M-MDSCs. It is known that c-FLIP is constitutively required for the development, survival and function of M-

MDSCs (Haverkamp, Smith et al. 2014). Therefore, we generated *in vitro* BM-MDSCs and after four days of differentiation, by immuno-magnetic sorting we isolated the M-MDSC subset ($CD11b^{+}/Ly6G^{-}/Ly6C^{high}$). These cells were then treated for 24 hours with the functional doses of eight different drugs (5-FU, Gemcitabine, Docetaxel, Paclitaxel, Oxaliplatin, Cisplatin, Etoposide and Irinotecan) and 5 treatments that were not effective in depleting the M-MDSCs during the differentiation (Cyclophosphamide, Fludarabine, Doxorubicin, Bevacizumab and Carboplatin). RNA was isolated from either treated or untreated M-MDSCs and we analyzed the expression of the two main splice variants of c-FLIP ($c\text{-}FLIP_{long}$ and $c\text{-}FLIP_{short}$) by real time PCR. As shown in Figure 2C and D, all the drugs that were effective in depleting the M-MDSCs during the *in vitro* differentiation induced a consistent down regulation of the two c-FLIP isoforms whereas the non-functional drugs did not affect the c-FLIP expression. These data highlight an important correlation between the expression levels of c-FLIP and the chemotherapy susceptibility in M-MDSCs.

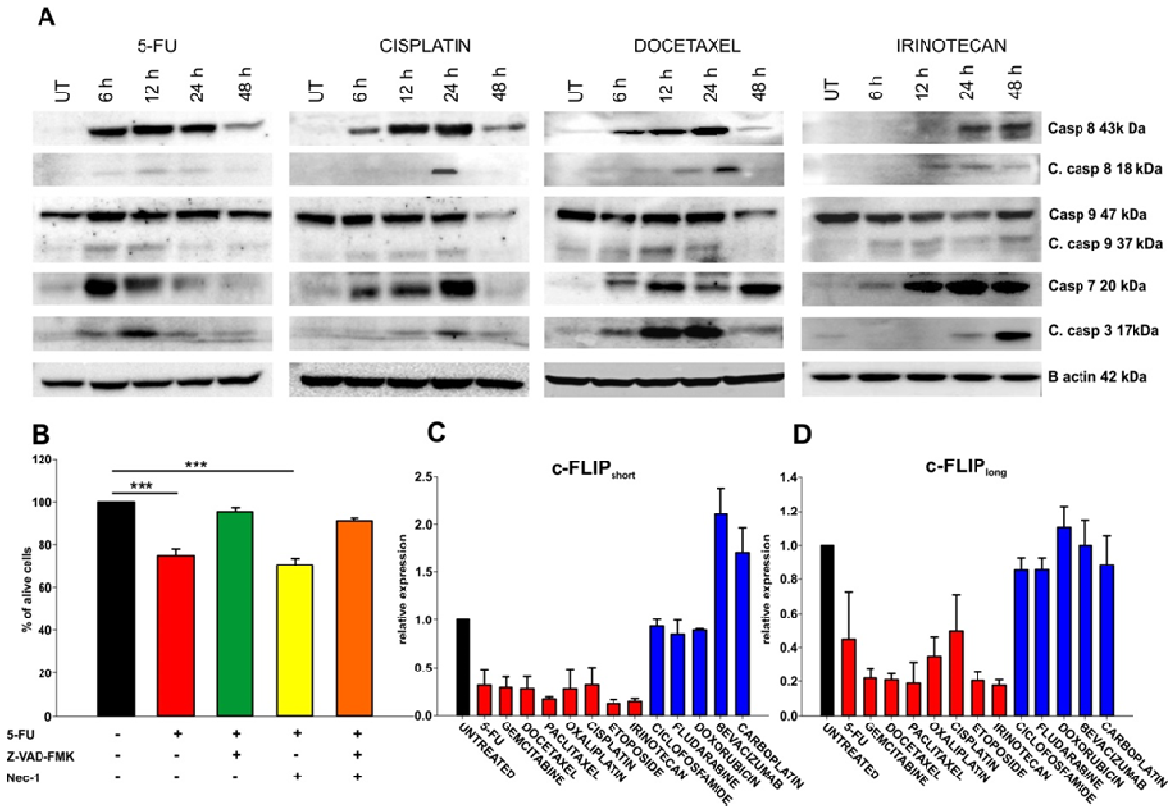

Figure 2. Chemotherapy treatment on MSC2 cell line induces the activation of the extrinsic apoptotic pathway. A) Western blot analysis of MSC2 cells treated with the functional doses of 4 pharmacological drugs (5-FU, Cisplatin, Docetaxel and Irinotecan). Although with different kinetics and intensity, a marked activation of the key caspases of the extrinsic apoptotic death pathway is detectable in all the treated samples compared to the untreated control, starting from 6 hours after the treatment. In particular, Caspase-8, Caspase-9, Caspase-7 (with anti-mouse antibodies able to recognize both the pro and cleaved caspases) and the cleaved Caspase-3. **B)** MSC2 cells were either untreated or treated with the functional dose of 5-FU in the presence of 50μM of Z-VAD-FMK, a pan-caspases inhibitor, or 50μM of Necrostatin-1 (Nec-1), which targets RIPK1 activity and blocks the necrotic cell death. Flow cytometric analysis of the vitality of the MSC2 cells showed that only the addition of the Pan Caspase Inhibitor (green bar) is effective in restoring the cell viability affected by the treatment with 5-FU, whereas no rescue is detectable when we treat the cells with Nec-1 (in yellow). **C)** M-MDSCs, immunomagnetically isolated from BM *in vitro* culture in presence of 40 ng/ml of both GM-CSF and IL-6 for four days, were treated for 24 hours with the functional dose of eight different pharmacological drugs (5-FU, Gemcitabine, Docetaxel, Paclitaxel Oxaliplatin, Cisplatin, Etoposide and Irinotecan) and five pharmacological treatments that were not effective in depleting the M-MDSCs during their *in vitro* differentiation (Cyclophosphamide, Fludarabine, Doxorubicin, Bevacizumab and Carboplatin). RNA was isolated from either treated or untreated M-MDSCs and the expression of the two main splice variants of c-FLIP (c-$FLIP_{long}$ and c-$FLIP_{short}$) was quantified by real time PCR.

3. c-FLIP enforced expression in human CD34$^+$ cells generates potent immune suppressor cells.

Based on our previously described data on mouse MDSCs, we decided to verify whether c-FLIP was also relevant for human MDSC survival and function. First, we demonstrated that human CD34$^+$ cells (obtained from leukapheresis of G-CSF-mobilized healthy donors) after four days of *in vitro* culture in the presence of 40 ng/ml of G-CSF and GM-CSF differentiated in more mature myeloid cells characterized by down-regulation of the CD34 marker, the expression of CD33 myeloid marker and powerful immunosuppressive function (Figure 3A), similarly to what previously described with whole BM cells (Solito S et al.,2011). Second, we used lentiviral expression vectors encoding the two major splice variants of human c-FLIP (c-FLIP$_{long}$ plus c-FLIP$_{short}$) to induce the expression of the target gene on human CD34$^+$ cells. As experimental control, we infected human CD34$^+$ cells with a lentivirus encoding luciferase. The infected cells were *in vitro* differentiated into MDSCs, as previously described. At day 4 of the *in vitro* culture, we added a single dose of 2.43 µg/ml of 5-FU, which we previously verified to eliminate selectively mouse M-MDSCs, in both c-FLIP- and luciferase-infected cells. At day 5, myeloid-cells were recovered and *in vitro* tested to evaluate their ability to control T cell proliferation. We demonstrated that c-FLIP up-regulation prevents chemotherapy-dependent loss of differentiated cells (CD34$^-$ cells) and preserves their immunosuppressive activity; in fact, 5-FU treated, c-FLIP-infected cells maintained their ability to arrest T cells proliferation compared to 5-FU treated, luciferase-infected cells (Figure 3B). These data provided evidence that the chemotherapy-induced MDSC immunomodulation is linked by c-FLIP. Furthermore and most importantly, these data suggest for the first time that c-FLIP up-regulation enhances the immunosuppressive ability of differentiated MDSCs opening a new biological role of this protein. Indeed, untreated c-FLIP- infected CD34$^+$ cells displayed and enhanced powerful suppressive activity, on a cell per cell basis, compared to untreated luciferase-infected cells (Fig. 3B).

To shed further light on this observation, $CD34^+$ cells were infected overnight with both the lentiviruses encoding $FLIP_L$- and c-$FLIP_S$ or luciferase (as control) and then co-cultured *in vitro* with activated, Cell-Trace-labeled T cells to quantify their immunosuppressive properties. As shown in Figure 4A, at all the different tested ratios of effector and target cells (0.5:1, 1:1, 2:1, 3:1) we detected a significant difference in the percentage of $CD3^+$ T cell recovery after the co-culture: c-FLIP-expressing $CD34^+$ cells (blue histograms) displayed an enhanced suppressive activity compared to controls (green histograms). To validate these data, we replicated the same experiment using a more differentiated, non-suppressive subset of human myeloid cells, i.e. $CD14^+$ monocytes freshly isolated from buffy coats of healthy donor. These cells were infected for four hours by spin-inoculation and, then co-cultured in presence of activated, Cell-Trace-labeled T cells. As shown in Figure 4B and C, also in this case, c-$FLIP_L$- and c-$FLIP_S$-infected $CD14^+$ cells displayed a significant increased suppressive ability compared to luciferase-infected $CD14^+$ cells. All these data suggest an unexpected and determinant role of c-FLIP to drive the immunosuppressive molecular program in myeloid cells.

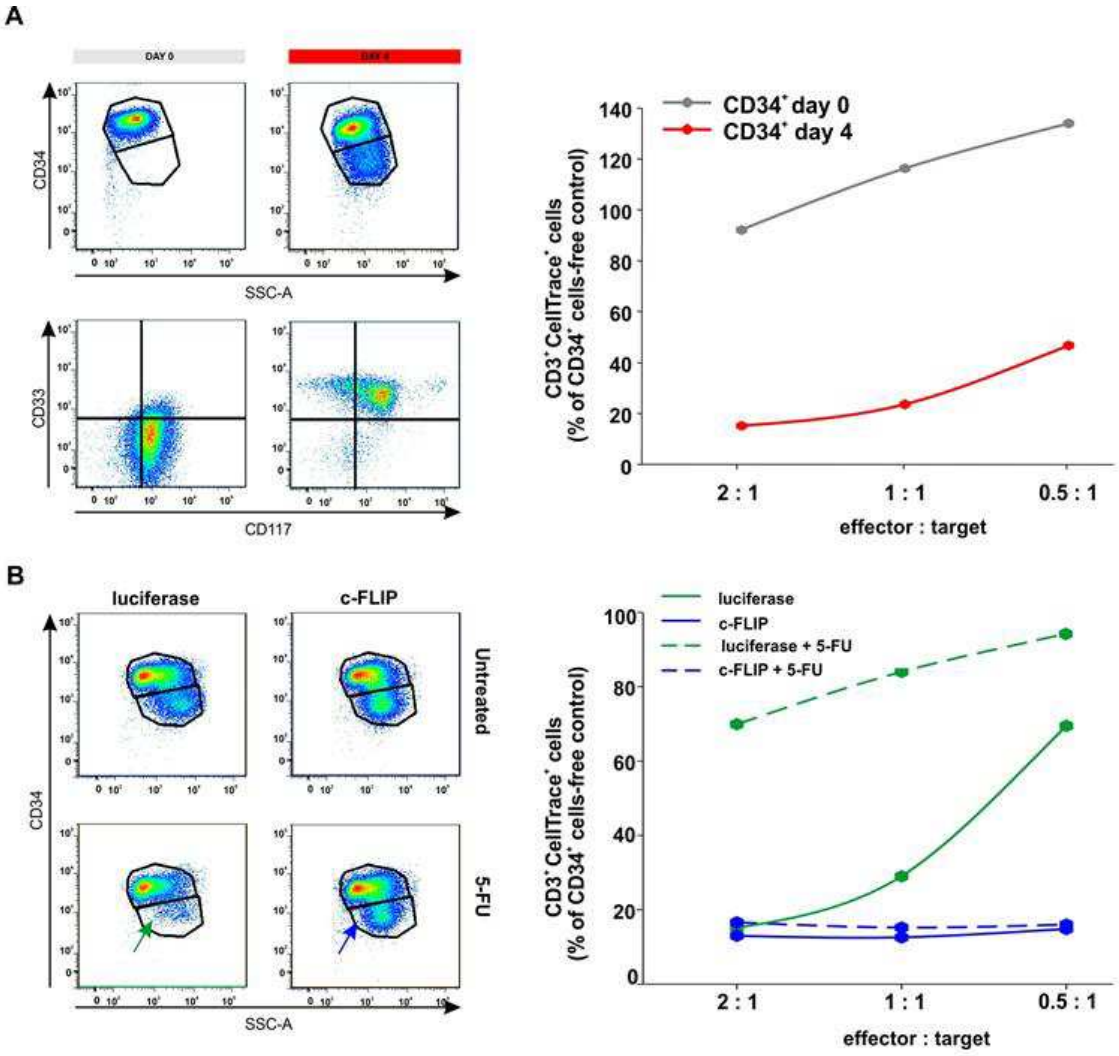

Figure 3. hCD34$^+$ differentiation process and chemotherapy treatment. A) hCD34$^+$ cells (obtained from leukapheresis of G-CSF-mobilized HDs) after four days of *in vitro* culture in the presence of 40 ng/ml of G-CSF and GM-CSF differentiated in more mature myeloid cells characterized by the down-regulation of the CD34 marker and the expression of CD33 marker and are characterized by greater powerful immunosuppressive function. (red line) compared to undifferentiated stem cells (grey line). **B)** hCD34$^+$ cells were infected overnight with lentiviral expression vectors encoding the two major splice variants of human c-FLIP (c-FLIP$_{long}$ plus c-FLIP$_{short}$). As experimental control, we infected human hCD34$^+$ cells with a lentivirus encoding luciferase. The infected cells were, later, *in vitro* differentiated into MDSCs by adding the cytokine cocktail previously described. At day 4 of the *in vitro* culture, we added 2.43 µg/ml of 5-FU, dose that is able to eliminate mouse M-MDSC, in both c-FLIP- and luciferase-infected cells. The flow cytometric analysis demonstrated that c-FLIP up-regulation prevents the chemotherapy-dependent loss of differentiated cells (CD34$^-$ cells) and control of immunosuppressive activity; in fact 5-FU treated, c-FLIP-infected cells (blue dotted line) maintained their ability to arrest T cells proliferation compared to 5-FU treated luciferase-infected cells (green dotted line). Moreover, untreated c-FLIP- infected CD34$^+$ cells (blue line) displayed powerful suppressive activity compared to untreated luciferase-infected cells (green line).

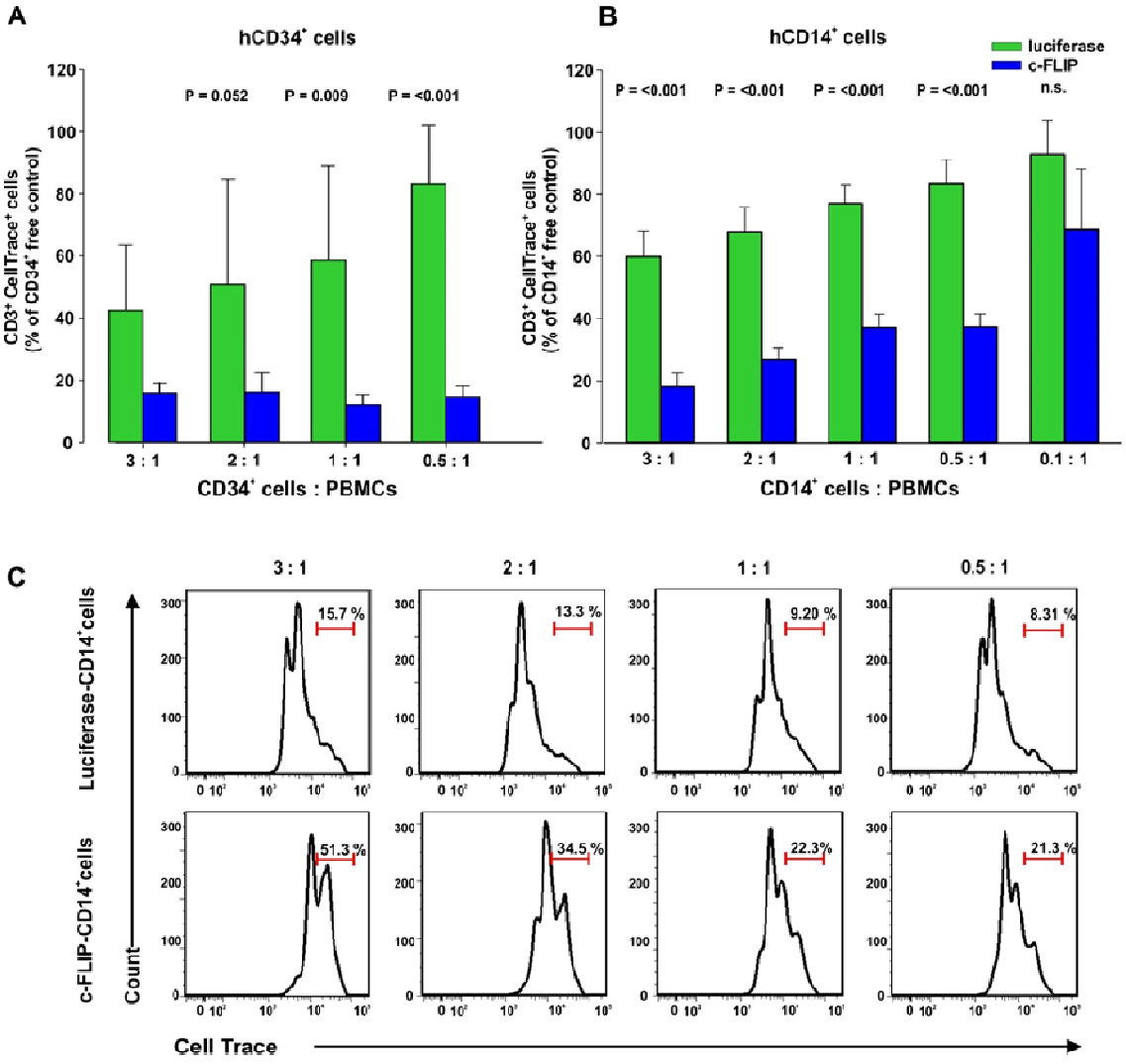

Figure 4. c-FLIP up-regulation by lentiviruses confers immunosuppressive properties to both immature and mature myeloid cells. **A)** Functional assay showed that, at different tested ratios of effector and target cells (0.5:1, 1:1, 2:1, 3:1), there was a difference in the percentage of $CD3^{+}$T cell recovery after the co-culture: c-FLIP-expressing $CD34^{+}$ cells (blue histograms) displayed a significantly augmented suppressive activity compared to controls (green histograms) (n= 5). **B)** $CD14^{+}$ monocytes freshly isolated from buffy coats of healthy donor are infected for four hours by spin-inoculation and tested for their suppressive ability. c-FLIP-infected $CD14^{+}$ cells (blue bars) displayed a significant increased suppressive ability compared to luciferase-infected $CD14^{+}$ cells (green bars; n= 7). **C)** Representative plots of a functional assay with luciferase- and c-FLIP infected monocytes.

4. Gene profile of c-FLIP-expressing CD14$^+$ monocytes and validation of up-regulated genes.

To investigate the molecular pathways triggered by c-FLIP enforced expression, we performed a transcriptome analysis of both c-FLIP- and luciferase- infected CD14$^+$ cells isolated from four different healthy donors (HDs). The supervised clustering (q.val <0.05; fold change >2) generated a list of more than 750 up-regulated genes in c-FLIP-infected monocytes compared to control (Figure 5A). In particular, these differentially expressed genes were significantly enriched in categories involved in inflammation, Notch-related pathway, IL-10-related pathway and NF-κB-related pathway. In details, c-FLIP enforced-expression promoted the over-expression of a list of MDSC-associated, immunosuppressive genes such as SOCS2, FAS, CCR7, CCL5, NF-κB, STAT3, CD38, PDL-1, PDL-2, IL4Rα, IL6, IL10, CFS3, PTGS2 and IDO1 (Figure 5B). Some of these putative c-FLIP-regulated genes were therefore tested and validated. By Real Time PCR we confirmed that c-FLIP-transduced monocytes did indeed upregulate both isoforms, i.e. c-FLIP$_L$- and c-FLIP$_S$, as well as IDO1, a key molecule for the immunosuppression of MDSCs. Moreover, by ELISA assay, we validated that c-FLIP-transduced CD14$^+$ cells secreted significantly higher IL-10 than luciferase-transduced CD14$^+$ cells. Finally, we verified that the enforced c-FLIP-expression on monocytes promoted an increased expression of specific surface markers by flow cytometry, such as PDL-2 (CD273), PDL-1 (CD274), CD80, CD163, CD44, CD38 and IL4Rα (CD124) (Figure 5C). All these data confirm that c-FLIP can affect the immunosuppressive molecular program of myeloid cells.

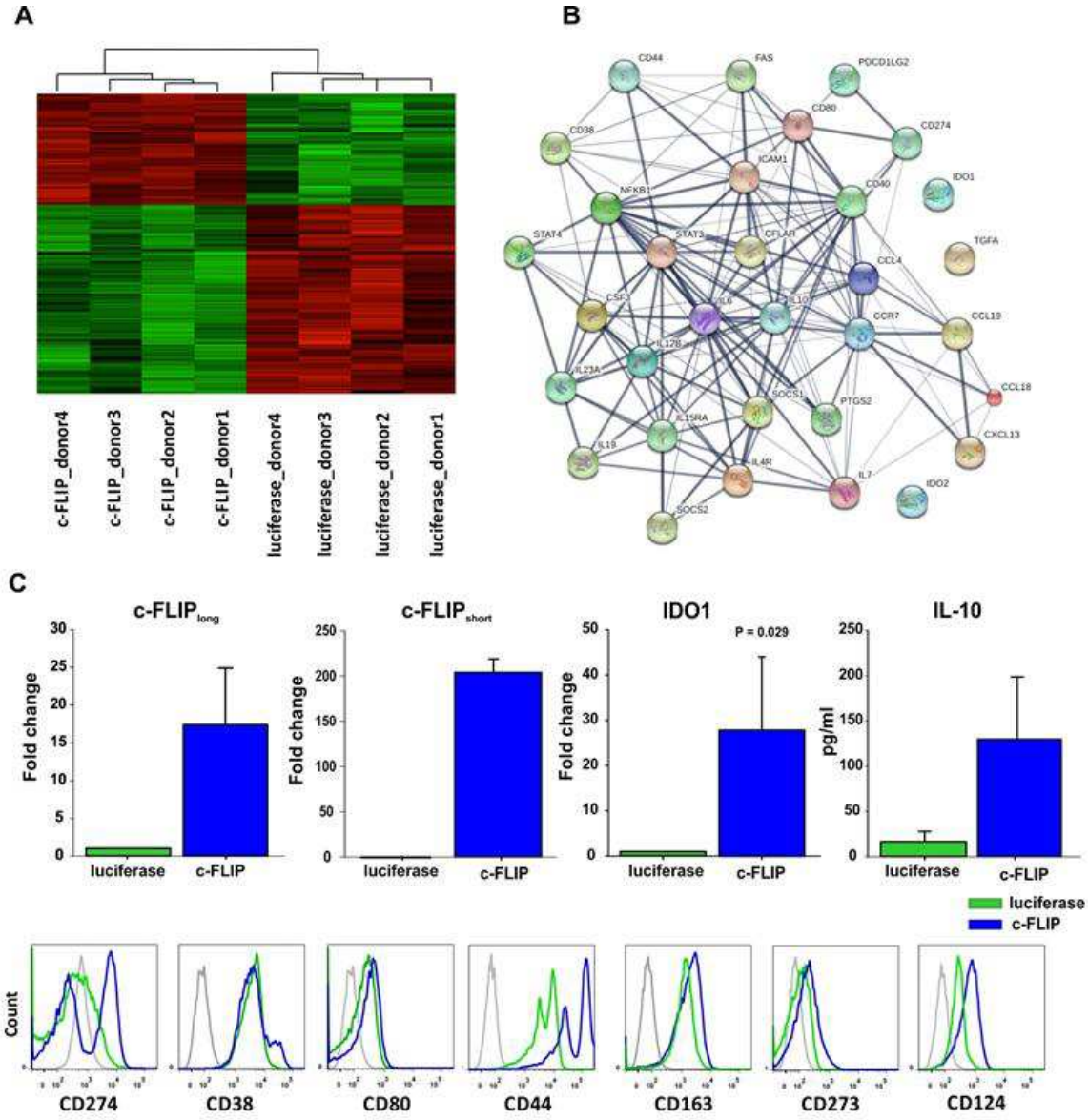

Figure 5. Molecular profiling defines a specific signature induced by c-FLIP up-regulation in monocytes. A) Cluster analysis comparing c-FLIP- with luciferase-infected monocytes. The analysis of the differentially expressed genes with q.val <0.05 and fold change >2 revealed 750 up-regulated and 1130 down-regulated genes. Sample and genes are clustered using Pearson correlation coefficient and average as distance metric and linkage, respectively. **B)** Functional protein association networks with STRING software of the immune-related genes up-regulated by c-FLIP-enforced expression. **C)** Validation by real time PCR of c-FLIP$_{long}$, c-FLIP$_{short}$ and IDO1 up-regulation. Quantification of the augmented production of IL-10 by c-FLIP-infeceted monocytes. Up-regulation induced by c-FLIP of specific surface markers by flow cytometry, such as CD273, CD274, CD80, CD163, CD44, CD38 and CD124.

5. c-FLIP-expressing monocytes control the progression of graft versus host disease (GvHD) in a xenogeneic experimental setting.

Allogeneic hematopoietic stem cell transplantation (HSCT) is the only treatment option for many hematological malignancies. The transplanted bone-marrow cells are both enriched for hematopoietic progenitors but also contain mature $CD4^+$ and $CD8^+$ T cells. These T cells promote the hematopoietic engraftment, reconstitute T-cell immunity (particularly in adults with reduced thymic function) and mediate a potent beneficial anti-tumor effect, known as graft versus leukemia (GVL). Unfortunately, donor T-cells can also cause graft-versus-host-disease (GvHD), a broad and aggressive attack against host tissues (Pasquini, Wang et al. 2010, Blazar, Murphy et al. 2012). A growing body of literature demonstrated that the administration of MDSCs together with BM transplantation was able to significantly inhibit GvHD lethality and was associated with a decreased proliferation and activation of donor T cells (Highfill, Rodriguez et al. 2010, Koehn, Apostolova et al. 2015). Starting from these studies, we decided to assess the therapeutic potential of $FLIP_L$- and c-$FLIP_S$ -infected $CD14^+$ monocytes to control the disease progression of mice developing GvHD in a setting of xenogeneic transplant. For this purpose, we intravenously (i.v.) injected sub-lethally irradiated, immunocompromised NOD.Cg-*Prkdcscid Il2rgtm1Sug*/JicTac (NOG) mice (6-7 weeks old) with 10^6 human PBMCs isolated from buffy coat of HDs. At day 21 after the initial PBMCs challenge, when the percentage of circulating human $CD3^+$ T lymphocytes was more or less the 5% of total blood cells, we treated the mice by the i.v. transfer of 10^6 c-FLIP- or luciferase-infected human monocytes. The treatment was repeated four times, on day 21, 28, 42 and 49 after the initial challenge and the mice were monitored for the immunological, clinical and histopathological GvHD scores. As shown in Figure 6A, the injection of c-FLIP-transduced $CD14^+$ cells (blue line), efficiently reduced GvHD development, resulting in significantly improved long-term survival ($P < 0.001$) compared to both untreated mice and mice that received luciferase lentivirus-infected $CD14^+$ cells. By day 33, after only two treatments, we performed flow cytometric analysis of the blood, spleen and bone marrow. As shown in the graphs B, C and D of Figure 6, after only two injections of $CD14^+$ cells, there is a lower

expansion of human $CD3^+$ T lymphocytes in all the three analyzed tissues in the c-FLIP animals, compared to the luciferase controls. Moreover, the analysis of the splenocytes after overnight stimulation with a cell stimulation cocktail (PMA and ionomycin), revealed that the mice treated with c-FLIP-transduced monocytes possessed functionally exhausted $CD8^+$ T cells compared to control mice, showing a significantly lower IFN-γ production (Figure 6E). Interestingly, in the peripheral blood of mice treated with c-FLIP-transduced $CD14^+$ cells, by day 53 after the initial PBMCs challenge, we could appreciate the expansion in a population of human Treg that was absent in the control mice (Figure 6F). In addition, histologic and immunohistochemistry (IHC) analysis of GvHD severity on day 70, revealed a lower GvHD histopathological score and lower percentage of human $CD3^+$ T cells in all analyzed organs, compared to the control mice. In particular, in all mice treated with luciferase-transduced, control monocytes the gastro-intestinal tract and skin presented severe lesions characterized by a consistent number of apoptotic bodies and a more diffuse immune infiltrate whereas the organs of mice treated with c-FLIP-infected monocytes were completely normal (Figure 6G). Therefore, we can conclude that the infusion of c-FLIP-expressing monocytes generated by lentivirus transduction has a great therapeutic impact on controlling the disease progression of GvHD.

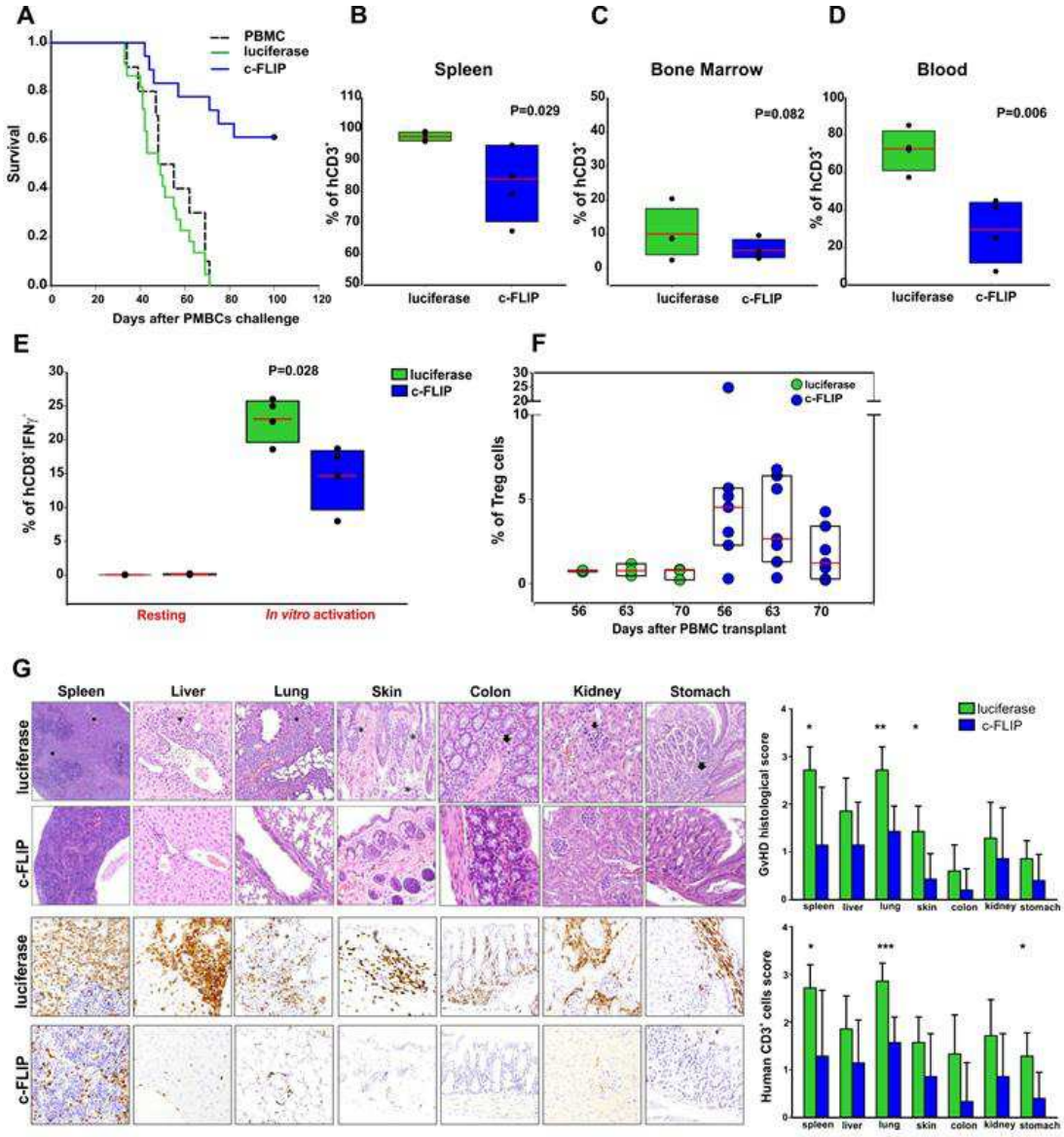

Figure 6 c-FLIP-infected monocytes mitigate the severity of GvHD pathology in a human model of xenogeneic GvHD disease in NOG mice engrafted with human PBMCs. Sub-lethally irradiated NOG mice were intravenously injected with 10^6 of human PBMCs. On day 21, 28, 42 and 49 mice were treated with 10^6 c-FLIP- or luciferase-infected $CD14^+$ monocytes. **(A)** Kaplan-Meier survival curve represents two independent experiments (PBMC n=8, luciferase n=15, c-FLIP n=12), P <0.001. **(B,C,D)** Human lymphocytes population analysis from tissues harvested at GvHD mice treated two times with c-FLIP- or luciferase- $CD14^+$ monocytes. Frequency of human $CD3^+$ lymphocytes in spleen **(B)**, bone marrow **(C)** and peripheral blood **(D)** of engrafted mice. **(E)** Percentage of IFN-γ- producing $CD8^+$ T cells in the spleen after overnight *in vitro* stimulation with PMA-IONO. **(F)** From day 55 in the peripheral blood of c-FLIP-infected- $CD14^+$ cells- treated mice is detectable a population of regulatory T cells (Treg) that is completely absent in the control mice. **(G)** Representative histological analysis of GvHD target organs in each group (n=3). Tissue harvest at day 70, were stained with hematoxylin eosin safran and evaluated for the histopathological score and immunohistochemistry staining for anti-human CD3 (0-3 point scale with 0 representing no involvement and no human- $CD3^+$ T cells and 3 reflecting severe impairment of the organ and a huge lymphocytes infiltration).

6. Rosa26.vFLIP knock-in mouse model.

In order to understand better the c-FLIP-dependent molecular pathways, we took advantage of a transgenic (Tg) mouse model expressing the viral form of FLIP (vFLIP): the Rosa26.vFLIP knock-in mice (Ballon, Chen et al. 2011, Ballon, Akar et al. 2015). vFLIP proteins are essential components of γ-herpesvirus such us equine herpesvirus-2 (EHV-2), herpesvirus saimiri (HVS), the Kaposi-associated human herpesvirus-8 (HHV-8), the rhesus rhadinovirus (RRV) and the human molluscipoxvirus (MCV). The structures of v-FLIP proteins are similar to c-$FLIP_S$, with the only exception that the two DEDs of c-$FLIP_S$ are followed by 20 amino acids that appear to be crucial for its ubiquitination and targeting for proteasomal degradation. The Tg mice expressing vFLIP were generated with the introduction of a cDNA-encoding FLAG-tagged vFLIP preceded by a loxP-flanked neo^R-STOP cassette and followed by frt-flanked IRES-EGFP sequence into the ubiquitously expressed ROSA26 locus. The elimination of the STOP cassette and the subsequent activation of Tg expression were achieved by crossing ROSA26.vFLIP knock-in mice with mice expressing Cre recombinase under the control of the endogenous Lyz2 promoter, thus resulting in mice with vFLIP expression in all myeloid cell lineage. EGFP co-expressed with vFLIP from a common transcript due to the insertion of an IRES sequence between the 2 gene sequences and was used to identify cells expressing the vFLIP Tg. Tg mice (carrying both Cre and vFLIP genes) were born at the expected Mendelian frequency but showed developmental abnormalities, a significant lower weight and all the mice died within four weeks (data not shown). As shown in Figure 7A, histologic analysis of the tissues of 4 weeks old vFLIP Tg mice revealed a massive infiltration of myeloid cells. In particular, a severe myocarditis was present in the heart muscle and the lung was characterized by a progressive interstitial pneumonia. In the liver, the infiltration is mostly condensed around the portal triad and the bile duct and in the pancreas of vFLIP Tg mice the endocrine component is completely destroyed and occupied by myeloid cells as well as the lamina propria of the gut lumen. Moreover, the spleen of the Tg mice displayed a complete alteration of its structure (Figure 7B): B ($B220^+$) and T ($CD3^+$) cells

were significantly replaced by mature myeloid cells ($CD11b^+$) at different stages of differentiation and we observed a sever extramedullary erythropoiesis. Surprisingly, Treg cells ($FOXP3^+$) number in the spleen was not affected.

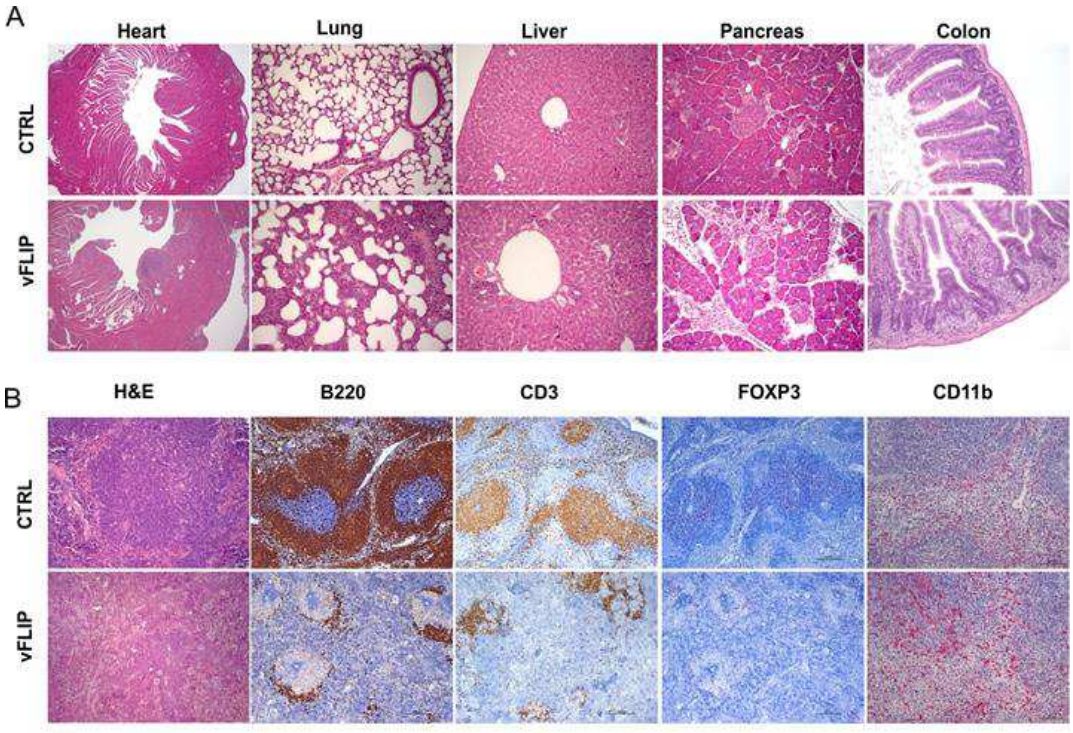

Figure 7. Characterization of vFLIP Tg mouse model
A) Representative histological analysis of heart, lung, liver, pancreas and colon of control and vFLIP Tg mice. Tissue were harvest from 4 weeks old mice and stained with hematoxylin eosin (H&E). **B)** The spleen of control and vFLIP Tg mice was stained with H&E and immunohistochemistry staining to detect B cells ($B220^+$), T cells ($CD3^+$), Treg cells ($Foxp3^+$), myeloid cells ($CD11b^+$).

Furthermore, the analysis of the sera of the vFLIP Tg mice disclosed a significant overproduction of cytokines and chemokines, including TNFα, IL-10, IL-6, IL-17, IL-18, G-CSF, RANTES, IL-1β compared to the control, suggesting again a marked inflammatory condition (Figure 8A). Flow cytometric analysis of the bone marrow revealed that Rosa26.vFLIP;LysMcre mice (in red) had an higher absolute number of $CD11b^+$ myeloid cells (Figure 8B) but a lower number of B cells ($B220^+$, Figure 8C) compared to the wild type (WT) littermate control mice that did not express CRE recombinase. In detail, both the M-MDSCs and PMN-

MDSCs were significantly augmented compared to the control (Figure 8D). Similar results were obtained in the peripheral blood and spleen (data not shown). Moreover, we isolated by fluorescence-activated cell sorting (FACS) monocytic ($CD11b^+$ $Ly6G^-$ $Ly6C^{high}$) and the PMN ($CD11b^+$ $Ly6G^+$ $Ly6C^{low}$) subsets from BM to test the immunosuppressive activity of these cells at their steady state.

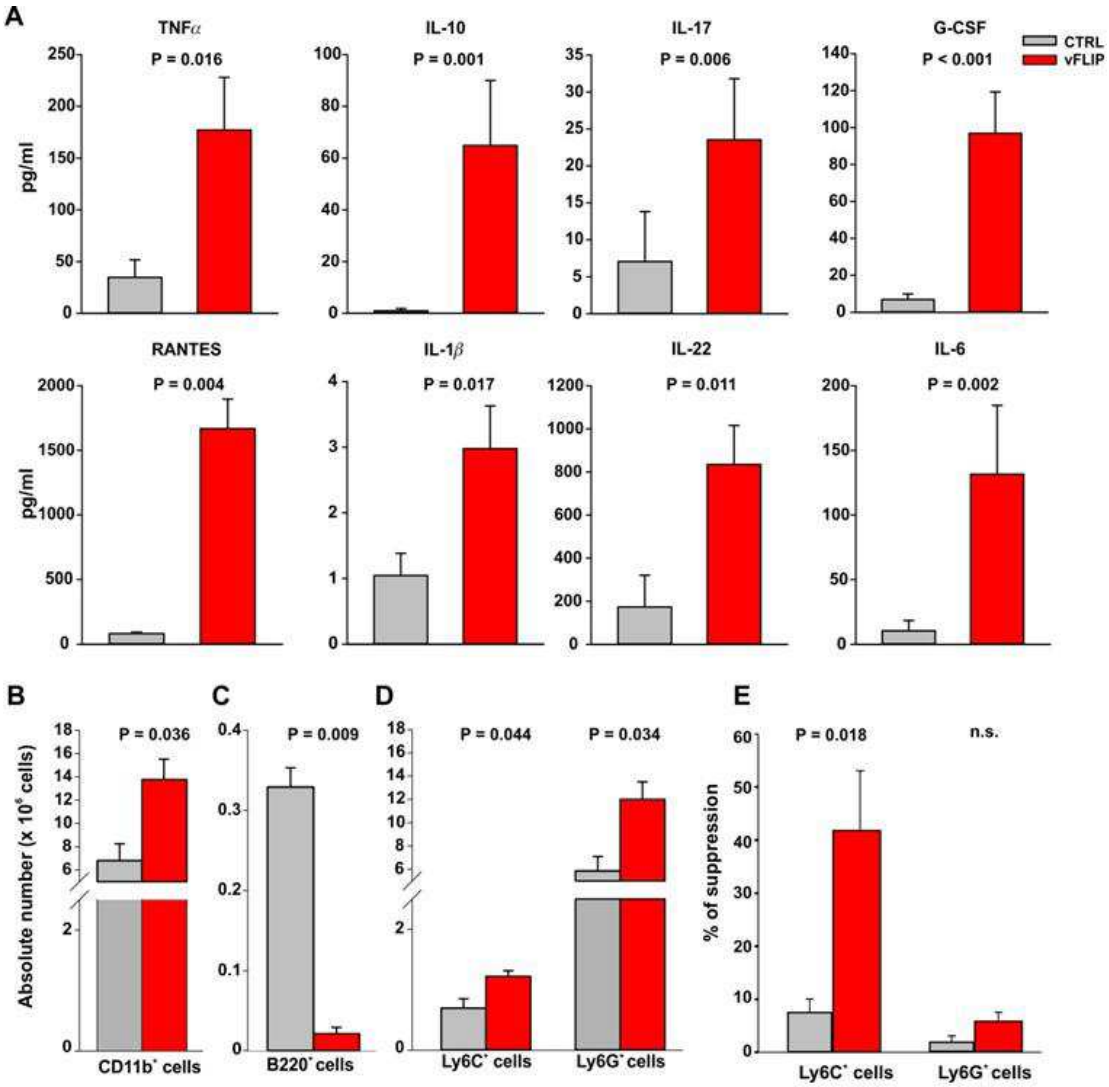

Figure 8. Characterization of vFLIP Tg mouse model

A) Perturbation of serum cytokines. 8 serum cytokines were analyzed in Rosa26.vFLIP;LysMcre$^+$ mice by ProcartaPlex Mouse Cytokine & Chemokine Panel 1A (36 plex) . Data are representative of 10 Tg and 10 control mice. **B)** Flow cytometric analysis of 5 mice per group displayed increase absolute number of $CD11b^+$ cells, decrease of $B220^+$ cells number **(C)** and an augmented number of both M-MDSCs and PMN-MDSCs **(D)**. **E)** Functional assay of isolated monocytic ($CD11b^+$ $Ly6G^-$ $Ly6C^{high}$) and the PMN ($CD11b^+$ $Ly6G^+$ $Ly6C^{low}$) cells: vFLIP-expressing monocytes cells displayed a significantly augmented suppressive activity compared to controls, whereas there is no difference in the functionality of the PMN- subset (n= 5). P-value derived from two-tailed unpaired Student's t-test on the means.

Interestingly, v-FLIP-expressing-monocytes possessed a significantly higher suppressive ability compared to the WT cells, whereas no difference in the functionality of the PMN- subset was highlighted (Figure 8E), suggesting that the increased expression of vFLIP on myeloid cells is sufficient to induce their immunosuppressive ability.

To confirm the augmented suppressive ability of the v-FLIP Tg monocytes we tested these cells *in vivo* in an adoptive cell therapy (ACT) experimental setting. Briefly, 7 weeks old C57BL/6J mice were s.c. injected with $1x10^6$ of EG7 tumor cells (EL-4 cells transduced with OVA). After 10 days (when tumor mass reached a volume of ~ 9 mm^3), tumor-bearing mice were injected i.v. with $0.5x10^6$ antigen-stimulated T lymphocytes specific for OVA class I epitope, previously expanded *in vitro* from C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-1 mice). These mice contain transgenic inserts for mouse Tcra-V2 and Tcrb-V5 genes encoding for a transgenic T cell receptor able to recognize the immunodominant ovalbumin residues 257-264 in the context of H2-K^b. After 2 hours, we transferred either WT or Rosa26.vFLIP;LysMcre$^+$ monocytes. Tumor volume was monitored every 3 days. Mice treated with ACT + Rosa26.vFLIP;LysMcre$^+$ monocytes (blue line) displayed the same tumor growth and survival rate of untreated mice, indicating that Rosa26.vFLIP;LysMcre$^+$ monocytes suppressed the effects of transferred, antigen-specific T lymphocytes mirroring the biological activity of functional MDSCs (Figure 9).

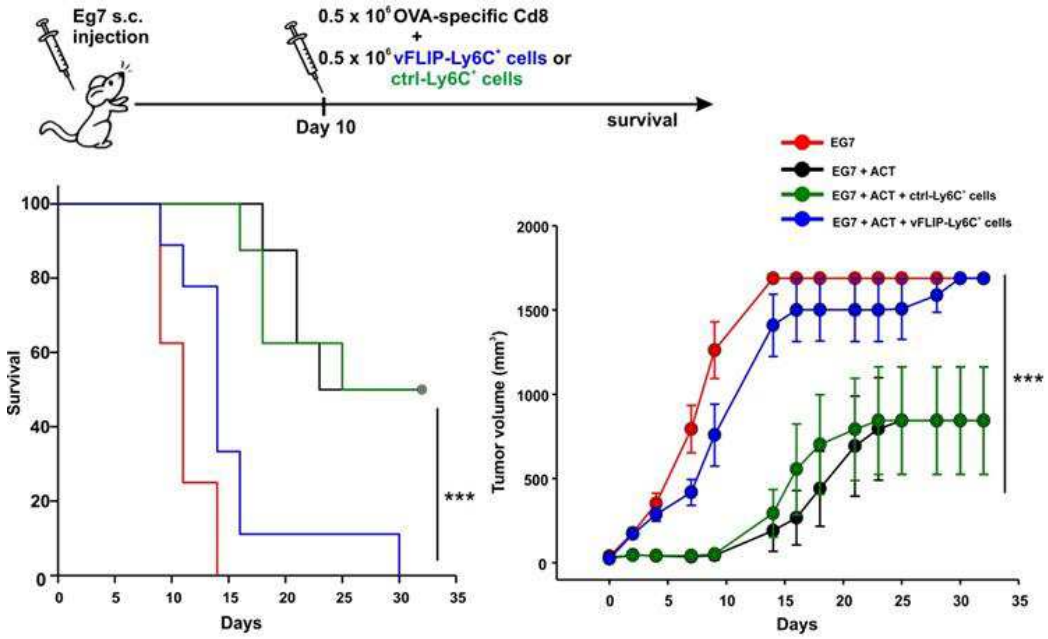

Figure 9. vFLIP- monocytes abrogate the therapeutic effect of ACT on tumor growth. 7 weeks old C57 mice were subcutaneously injected with $1x10^6$ of EG7 tumor cells. After 10 days (tumor volume ~9 mm^3) mice were injected intravenously with $0.5x10^6$ antigen-stimulated T lymphocytes specific for OVA class I epitope and, after 2 hours, we transfer or wt- or Rosa26.vFLIP;LysMcre-monocytes ($CD11b^+Ly6C^+$ cells). Tumor volume was monitored every 3 days. Mice treated with ACT + Rosa26.vFLIP;LysMcre- $CD11b^+Ly6C^+$ cells (blue line) displayed the same tumor growth rate and survival of untreated mice (red line).

7. vFLIP$^+$ M-MDSCs are resistant to chemotherapy.

To investigate the correlation between the level of FLIP and chemotherapy susceptibility in M-MDSCs, we took advantage of the Rosa26.vFLIP;LysMcre mouse model. For this purpose, as previously described, we added the functional doses of 4 chemotherapeutics (5-FU, Cisplatin, Etoposide, Docetaxel) at the beginning of WT and Tg BM cell culture of differentiation. After 4 days, flow cytometry analysis of the CD11b$^+$/Ly6G$^-$/Ly6C^{high} and CD11b$^+$/Ly6G$^+$/Ly6C$^{low/int}$ cell subsets, showed a significantly lower contraction in M-MDSCs of Tg-derived compared to WT-derived cultures (Figure 10A). Moreover, at the end of the *in vitro* culture, an higher percentage of viable CD11b$^+$Ly6C$^+$GFP$^+$ cells were detectable in chemotherapy-treated MDSCs compared to untreated controls (Figure 10B). Finally, we compared the effect on the immune suppressive ability of the BM-MDSCs after the pharmacological treatment and demonstrated that the expression of vFLIP in myeloid cells partially prevented the chemotherapy-dependent loss of immunosuppression (Figure 10C).

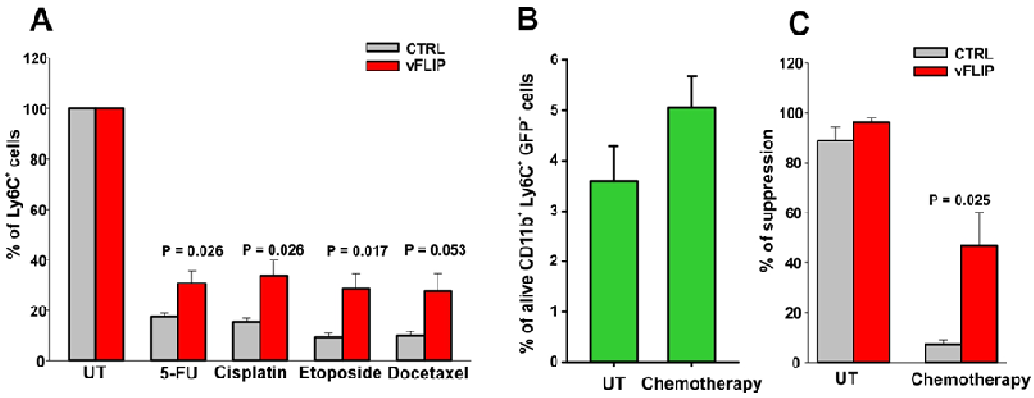

Figure 10. vFLIP-expressing M-MDSCs are protected from chemotherapy. The functional doses of 4 chemotherapeutics (5-FU, Cisplatin, Etoposide, Docetaxel) were added at the beginning of the culture of differentiation of both wt and Tg BM cells in presence of 40 ng/ml of GM-CSF and IL-6 for four days. After 4 days, flow cytometry analysis of the CD11b$^+$/Ly6G$^-$/Ly6C^{high} and CD11b$^+$/Ly6G$^+$/Ly6C$^{low/int}$ cell subsets, showed a significant lower contraction of the M-MDSCs in the Tg-derived culture compared to wt **(A)** and an higher percentage of alive CD11b$^+$ Ly6C$^+$ GFP$^+$ cells were detectable in chemotherapy treated MDSC compared to untreated control **(B).** Treated-BM-MDSCs derived from Tg mice partially maintained the suppressive ability after the chemotherapy treatment **(C).**

DISCUSSION

The clinical inefficiency of many current anti-tumor treatments is mainly due to the presence of tumor-driven microenvironment, which promotes the expansion of myeloid cells characterized by immunosuppressive functions named MDSCs. MDSCs are a heterogeneous population of myeloid cells comprising cells at various stages of differentiation. MDSCs prevent the activation and function of T lymphocytes, limiting the success of immunotherapy strategies aimed at eradicating developing cancer cells. In mice, the heterogeneity between the two main subsets of MDSCs, the M-MDSC and the PMN-MDSC, has been reported to occur from a diverse activation of the apoptotic pathways: PMN-MDSCs require the anti-apoptotic molecule MCL-1 for their development; in contrast, M-MDSC generation and survival constitutively require the presence of the anti-apoptotic protein c-FLIP (Haverkamp, Smith et al. 2014). An additional implication of the requirement for c-FLIP expression in M-MDSCs and their precursors comes from *in vivo* use of chemotherapy drugs. Several chemotherapy agents cause a selective depletion of monocytic cells, including 5-FU, gemcitabine and trabectedin (Ugel, Peranzoni et al. 2012, Germano, Tinessa et al. 2013). Chemotherapy drugs may affect c-FLIP expression in monocytic cells, both directly and indirectly. In mice, MDSC elimination is sufficient to restore the efficacy of immune intervention and restrain tumor progression (Ugel, Peranzoni et al. 2012). In this work, we demonstrated that low doses of different chemotherapies, belonging to classes endowed with different mechanism of action, affect MDSC *in vitro* differentiation from BM progenitors, causing a contraction of M-MDSCs, thus resulting in a complete loss of immunosuppression. Moreover, we demonstrated this chemotherapy-induced MDSC contraction both in human and mouse experimental setting, allowing us to elucidate a shared molecular pathway in response to therapy. In fact, we demonstrated that the final end point of M-MDSC depletion is mediated by the activation of the caspases in the extrinsic apoptotic pathway and down-regulation of c-FLIP expression. The balance among myeloid cells and lymphocytes and, in particular, the decline in immunosuppressive myeloid cells

within the tumor microenvironment appears to be fundamental for successful implementation of immunotherapy and improved clinical efficacy. As reported by Welters and colleagues, the treatment of tumor-bearing mice with chemotherapy and therapeutic vaccination resulted in superior survival and was directly related to chemotherapy-mediated altered composition of the myeloid cell population in the blood and the tumor (Welters, van der Sluis et al. 2016). Since a clinical trial is already under way to test this (NCT02128126), our data highlight c-FLIP as very promising biomarker for MDSC enumeration and classification. This might be a clinically relevant parameter since bridges the influence of tumor cells on the accumulation of c-FLIP-expressing cells and might correlate with the cancer patient's response to chemotherapy. Therefore, in the next future we plan to enumerate circulating MDSCs by combining surface markers with c-FLIP intracellular staining. We plan to quantify the endogenous level of c-FLIP expression in myeloid cells of patients with different tumors and, by comparing to HDs, monitor its expression modulation in response to chemotherapy. The expression of c-FLIP would allow the identification of those patients that are more likely to benefit from conventional chemotherapy or immunotherapy approaches in most appropriate tumor phase to guarantee the best anti-tumor response.

In this study, we also demonstrated that c-FLIP is a driving molecule in MDSC biology, suggesting, for the first time, a new and unanticipated role of this protein as regulator of the immune-suppression. We reported that the enforced expression of c-FLIP by lentiviral infection in $hCD34^+$ cells and $hCD14^+$ cells generates human myeloid suppressors cells able to inhibit T cell activation and proliferation both *in vitro* and *in vivo*. We assessed *in vivo* the immunosuppressive functions of c-FLIP-infected monocytes to control GvHD severity. The intravenous injection of c-FLIP-infected monocytes efficiently reduced the GvHD progression, resulting in improved long-term survival of human PBMC engrafted mice. Despite improvements in our understanding of transplant immunology and clinical/supportive care, GvHD remains a clinical challenge and a major cause of morbidity and mortality for allogeneic hematopoietic stem cell transplantation (HSCT) recipients (Pasquini, Wang et al. 2010, Blazar, Murphy et al. 2012).

GVHD is initiated by the host APCs, which activate T cells by antigen-presentation and promote a strong and uncontrolled storm of pro-inflammatory cytokines such as IFN-γ, TNF-α and TGF-β (Blazar, Carreno et al. 2003, Socie and Blazar 2009). These immune mediators maintain a positive feedback loop between cytokines and immune cells that result in end-organ damage, which is clinically recognized as acute GvHD in the skin, lungs, gut and liver. The resulting tissue damage, if not treated, will further amplify the process to more severe stages of GvHD pathology, which are extremely difficult to control (Shlomchik, Couzens et al. 1999, Matte, Liu et al. 2004). Systemic corticosteroids and calcineurin inhibitors remain the standard, primary therapy for GvHD (Garnett, Apperley et al. 2013). Therefore, novel and improved therapies are urgently needed. To this purpose, we suppose that the use of immune modulatory therapy based on the injection of c-FLIP-expressing monocytes could be a very promising strategy. For the clinic translation, we plan to convert our strategy based on monocyte infection by c-FLIP expressing lentiviruses with a safer therapeutic procedure based on the transduction of monocytes with naked RNA encoding c-FLIP. Feasibility and safety of administration of naked RNA have been shown in preclinical and clinical settings (Weide, Carralot et al. 2008, Kranz, Diken et al. 2016, Hinz, Kallen et al. 2017). Therefore, we have already filed an European patent application (PCT/EP2017/051068) in collaboration with Biontech (Appendix 1) to finalize the clinical translation of our approach.

However, in order to translate our strategy to generate suppressor cells in a clinical setting, a deeper understanding of c-FLIP in MDSCs biology would be mandatory. Therefore, we performed a transcriptome analysis of c-FLIP-infected monocytes isolated from HDs and related controls. The supervised clustering generated a list of more than 750 up-regulated genes, which enriched in categories involved in inflammation pathways, Notch- and IL-10-associated pathway. Many well-known genes related to immune regulation were up-regulated, such as IDO1, IDO2, CD274, CD273, CD38, IL-10, 1L-6, CCR7, CD44 and CD124. These findings create a first link between c-FLIP and suppressive program in MDSCs. Further studies are now in progress to unveil how c-FLIP induces and controls the activation of the suppressive pathway. Epigenomic profiling of c-FLIP-induced

chromatin changes and the identification of the transcription factors interacting or depending upon c-FLIP activation are necessary to dissect c-FLIP network of molecular interactions and shed more light on the suppressive circuits.

Finally, to have a complete picture of the *in vivo* relevance of the data, we took advantage of a Tg mouse model that expresses the viral form of c-FLIP (vFLIP): the Rosa26.vFLIP knock-in mice. These mice were bred by crossing with mice expressing Cre recombinase under the control of the endogenous Lyz2 promoter, thus resulting in mice with vFLIP expression in myeloid cell lineage. As c-FLIP, it is well established that also vFLIP has an anti-apoptotic activity (Sun, Matta et al. 2003, Efklidou, Bailey et al. 2008) and plays an important role in the pathogenesis of Kaposi sarcoma herpesvirus (KSHV, or human herpesvirus-8) associated tumors. KSHV is the etiologic agent of three tumors: the Kaposi sarcoma (KS), primary effusion lymphoma (PEL) and a form of multicentric Castleman's disease (KSHV-MCD) (Djerbi, Screpanti et al. 1999, Guasparri, Keller et al. 2004, Ballon, Chen et al. 2011). In particular, it was previously demonstrated that mice with vFLIP expression in endothelial cells display a profound pro-inflammatory phenotype with severe perturbation of serum cytokines, similarly to KSHV-inflammatory cytokine syndrome (KICS), as well as the expansion of myeloid cells. In this context, endothelial vFLIP expression might trigger a cascade of events leading to changes in host microenvironment, ultimately favoring immune evasion, angiogenesis and tumor progression during KSHV pathogenesis (Ballon, Akar et al. 2015). In our model, the expression of vFLIP in myeloid cells induced systemic changes and pathological abnormalities: although born at the expected Mendelian frequency, mice showed a significant lower weight and died within four weeks. We observed a remodeling of the immune tissue composition: vFLIP-Tg mice displayed a contraction in B cells and an augmented absolute number of myeloid cells, both monocytic and PMN subsets. Interestingly, only the monocytic population displayed suppressive activity *ex vivo* compared to the control. This suppressive ability was also confirmed *in vivo* in an ACT experimental setting, in which v-FLIP-Tg mouse-derived monocytes suppressed the effects of transferred antigen-specific T lymphocytes, mirroring the biological activity of tumor-induced MDSCs.

Furthermore, we detected in vFLIP mouse sera an overproduction of pro-inflammatory cytokines, including TNFα, IL-10, IL-6, IL-17, IL-18, G-CSF, RANTES, IL-1β, a cytokine profile overlapping with the one described for MCD and KICS. Finally, we demonstrated that also vFLIP has a protective role in monocytes against chemotherapy: indeed, vFLIP-enforced expression in myeloid cells partially prevented the chemotherapy-dependent apoptosis and loss of immunosuppression. Taken together, all these data highlight for the first time FLIP proteins as key regulators in MDSC biology and function.

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APPENDIX

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Acknowledgement of receipt

We hereby acknowledge receipt of your request for the processing of an international application according to the Patent Cooperation Treaty as follows:

Submission number	4968106
PCT application number	PCT/EP2017/051068
Date of receipt	19 January 2017
Receiving Office	European Patent Office, The Hague
Your reference	674-208 PCT
Applicant	BIONTECH AG
Number of applicants	2
Country	DE
Title	ENGINEERED CELLS FOR INDUCING TOLERANCE
Documents submitted	eolf-pkda.xml eolf-appb.xml eolf-fees.xml eolf-appb-P000001.pdf (56 p.) eolf-appb-P000003.pdf (1 p.) eolf-requ.xml eolf-seql.txt eolf-vlog.xml eolf-appb-P000002.pdf (5 p.) eolf-appb-P000004.pdf (10 p.)
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Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

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