Differential Induction of Ly6G and Ly6C Positive Myeloid Derived Suppressor Cells in Chronic Kidney and Liver Inflammation and Fibrosis

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Abstract
CD11b+Gr1+ myeloid derived suppressor cells (MDSC) are known to be very potent suppressors of T cell immunity and can be further stratified into granulocytic MDSC and monocytic MDSC in mice based on expression of Ly6G or Ly6C, respectively. Here, using these markers and functional assays, we aimed to identify whether MDSC are induced during chronic inflammation leading to fibrosis in both kidney and liver and whether additional markers could more specifically identify these MDSC subsets. In an adenine-induced model of kidney inflammation/fibrosis suppressive Ly6Gpos MDSC were induced. The suppressive function within the Ly6G+ MDSC population was exclusively present in IFNγRβ expressing cells. In contrast, in chronic inflammation in the liver induced by bile duct ligation, suppressive capacity was exclusively present in the Ly6Cpos MDSC subset. Gene expression analyses confirmed the differential origins and regulation of those MDSC subsets. Additionally, depletion of MDSC in either kidney or liver fibrosis enhanced fibrosis markers, indicating a protective role for MDSC in organ fibrosis. Thus, our data demonstrate that during liver inflammation and kidney fibrosis MDSC with similar function arise bearing a distinct marker profile and arising from different cell populations.

Introduction
Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of cells with a myeloid origin. Murine MDSC are generally CD11b and Gr-1 positive [1-3] and can mediate suppression via several mechanisms (Arginase-1, iNOS, ROS) [4] MDSC are described to exert
immunosuppressive function in cancer [10, 11], acute and chronic infections [12, 13]), under chronic inflammatory conditions [2], but also in autoimmune diseases [1]. Multiple inflammatory mediators such as IFNγ, TLR ligands [2], TNF [3], PGE2 [4, 5], S100 [6, 7], IL-1β [8] and IL-6 [9] have been described to induce, accumulate or activate MDSC, which then suppress T cell responses [10], modulate the cytokine expression by macrophages [11] or impair DC development [6]. Especially the role of IFNγ on the function and development of MDSC is discussed controversially. Whereas some publications show that the development of MDSC is IFNγ-dependent and that IFNγ is needed for the ROS or NO production [12, 13], other studies, in which MDSC development still occurred in IFNγR-deficient mice, suggest that IFNγ is not essential [10].

Organ fibrosis is a result of chronic inflammation and is accompanied by the infiltration of pro-inflammatory monocytes, macrophages, neutrophils and T cells. These inflammatory conditions go hand in hand with wound healing processes, which lead to continued replacement of dying parenchymal cells with connective tissue or extracellular matrix [14]. Organ fibrosis leads to severe functional damage of the organ and is one of the leading reasons for morbidity and mortality with growing prevalence in end-stage liver or kidney disease. During chronic inflammation many factors (e.g. IL-1β, TNF, IFNγ, DAMPs) are released, which may promote accumulation, activation or induction of MDSC in the inflamed organ [15]. These MDSC may then prevent immune-mediated damage and reduce the harmful effects of prolonged inflammation by switching off pro-inflammatory immune cells. However, specific identification of MDSC in chronically inflamed fibrotic organs is challenging, as pro-inflammatory monocytes, neutrophils and macrophages, expressing similar markers and effector molecules but lacking suppressive function, also infiltrate the inflamed tissue. In addition to their suppressive function, MDSC can be subdivided into two major populations that either express Ly6C or Ly6G [1, 16]. More specifically, monocytic Ly6Cpos MDSC express CD11b, Gr-1, Ly6C but no Ly6G and the granulocytic/neutrophilic Ly6Gpos MDSC express CD11b, Gr-1, Ly6G, but are low in Ly6C [15]. Many additional markers, such as B7H1, IL4Rα or IFNγRβ, are suggested to more specifically identify MDSC [17]. Myeloid cells stratified according to these markers can fulfill distinct suppressive functions in different diseases such as cancer, infection or autoimmunity [1, 18, 19]. However, it is not clear if these MDSC subpopulations play a suppressive role in organ fibrosis due to chronic inflammation.

In the kidney a suppressive role for MDSC has been described in renal cell carcinoma and renal transplantation ([20, 21]), but their role in kidney fibrosis has not been addressed so far. In the liver MDSC are known to accumulate in mouse models of acute immune-mediated liver injury ([22]) as well as in patients with chronic inflammatory liver disease, like hepatitis C ([23]), or hepatocellular carcinoma ([24]), which is thought to arise in part as a result of chronic liver inflammation and fibrosis ([25]). A well-established experimental animal model for liver fibrosis is bile duct ligation (BDL) in rodents, in which hydrophobic bile acid mediated liver injury leads to chronic inflammation, fibrosis and ultimately hepatic cirrhosis. In adenine-induced tubulointerstitial nephritis, excessive insoluble adenine causes tubular cell damage also leading to chronic inflammation and end-stage fibrosis. In this study, we aimed to identify the MDSC subsets that arise during chronic inflammation leading to fibrosis in both the kidney and the liver and further investigated whether additional markers could more specifically identify these MDSC subsets. Here, we describe that during adenine-induced tubulointerstitial nephritis suppressive capacity resides within the Ly6Gpos MDSC subset that also expresses IFNγRβ. In contrast, during chronic hepatic inflammation after bile duct ligation, suppressive capacity was exclusively present in the Ly6Cpos MDSC subset and did not correlate with IFNγRβ expression. These data indicate that during liver and kidney fibrosis suppressive MDSC with similar function, but different phenotype are induced.
Material and Methods
Mice, diet, treatments

C57BL/6J mice were bred in the central animal facility in Bonn according to the Federation of European Laboratory Animal Science Association guidelines and maintained under SPF conditions. Mice were fed an adenine-enriched diet (10g/5kg) (Sniff, Soest, Deutschland) to induce kidney fibrosis [26]. Mice were injected with $10 \mu$g LPS and $1 \mu$g IFN$\gamma$ every second day for 2–3 times to induce MDSC in the spleen [2]. Retinoic acid (Tretinoin, Roche, Grenzach-Wyhlen, Germany) was provided at 1g/liter in the drinking water of mice from day 7 onwards after bileduct ligation or from day 7 on after the start of adenine feeding. This corresponds to a daily dose of approx. 0,5 mg/mouse. All efforts were taken to minimize suffering. Mice were sacrificed by cervical dislocation. All animal experiments were approved by the Animal Care Commission of Nordrhein-Westfalen (84–02.04.2013.A014 and 84–02.04.2013.A129).

Bile duct ligation

Preoperatively, mice were injected with 5mg/kg carprofen. Mice were anesthetized by isoflurane inhalation during surgery. The abdomen was opened by a midline laparotomy and the bile duct was carefully mobilized and ligated with 5–0 silk. In sham-operated controls, the bile duct was mobilized but not ligated. The incision was closed with 5–0 silk. Mice were sacrificed at the indicated times after surgery and non-parenchymal liver cells were isolated and analyzed by flow cytometry.

Isolation of non-parenchymal liver and kidney cells

Isolation of liver non-parenchymal cells was performed as described before [27]. Shortly, livers were perfused and mechanically dissociated. Non-parenchymal cells were collected from the interface after density centrifugation. Cells were analysed by flow cytometry or sorted using the gating strategy as indicated. Isolation from the kidney was performed as described earlier [28]. Briefly, kidneys were perfused, mechanically dissociated and digested in RPMI 1640 (Gibco, Life Technologies, Darmstadt, Germany) containing collagenase and DNase. After letting tubular cells sediment for some minutes from homogenized cell suspensions the supernatant with non-parenchymal cells was taken to perform flow cytometry analysis or sort using the gating strategy as indicated.

Generation of bone marrow-derived MDSC

Bone marrow was flushed from tibias of C57BL-6 mice and cultured in alpha MEM w/o L-Glutamine (Lonza, Belgium) supplemented with 10% FCS (PAA), 1mM sodium pyruvate (Biocrom AG), 2mM L-glutamine (Gibco, life technologies), 100U/ml Penicillin, 100$\mu$g/ml Streptomycin (Gibco, life technologies), 0,05 mM 2-mercaptoethanol (Gibco, life technologies) and 200 U/ml (= 40 ng/ml) CSF-2 (GM-CSF) (Peprotech, USA) in petri dishes. After 4 days of culture cells were analysed by flow cytometry or sorted for in vitro suppression assays.

Real-time PCR

Liver and kidney tissue samples were homogenised and total RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel, Düren)). RNA was transcribed into cDNA using the High-Capacity CDNA Reverse Transcription kit (Gibco, life technologies) following the manufacturers instructions. Real-time PCR was performed using the SybrGreen PCR Master mix on a light cycler 480 instrument II (Roche, Switzerland) Primers used: $\alpha$-SMA fw: 5’-TCCAGAGTC-CAGCACAATACCAGT-3’, rv: 5’-TGACAGAGGACACTGAACC-3’, TGF-β fw:
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T cell suppression assay
MACS-purified CD8 T cells were labelled with 0.1μM carboxyfluorescein-succinimidyl-ester (CFSE) and stimulated with Dynabeads Mouse T-activator CD3/CD28 for T-Cell expansion and activation (Life Technologies, Darmstadt, Germany). Different subpopulations of sorted myeloid cells were cocultured with the T cells at the indicated ratios. Proliferation of T cells was analysed after 72h by flow cytometry and was based on the CFSE dilution.

Flow cytometry and sorting
The phenotype of myeloid cells was determined by multi-colour flow cytometry using the following antibodies: anti-CD11b (M1/70), anti-Ly6C (4K1.4), anti-Ly6G (1A8), anti-CD11c (N418), anti-F4/80 (BM8), anti-Ly-6C-E (M5/114.15.2), anti-CD34 (RAM34), anti-CD64 (X54-5/7.1), anti-CD86 (GL-1), anti-CD80 (16-10A1), anti-IFNγRβ (MOB-47), anti-IL-4Rα (I015F8), anti-CD62L (MEL-14), anti-B7–H1 (MIH-5) and CD45.2 (104). Antibodies were purchased from eBioscience or Biolegend. Dead cells were excluded with Hoechst-33258 (Sigma). All stainings were performed in the presence of 10 μg/ml Fc-Block (clone 2.4G2). Iso-type matching fluorochrome-labelled antibodies were used as indicated. For the enumeration of total cell numbers per organ an equal amount of counting beads was added to each sample. Samples were acquired using an LSR Fortessa (BD Bioscience, Heidelberg, Germany), cell sorting was performed with Aria III (BD Bioscience, Heidelberg, Germany), and data was analysed using FlowJo software (TreeStar, Inc, Ashland, OR).

Inflammatory gene expression analysis
Non-parenchymal cells were isolated from individual livers and kidneys 14 and 10 days after BDL and the start of adenine feeding, respectively. Cells were stained with antibodies against CD11b, Ly6G and Ly6C in the presence of Fc-block (clone 2.4G2). Cells were washed and subsequently sorted into CD11b+Ly6C+ and CD11b+Ly6G positive fractions from each organ. 20,000 cells were analysed for their inflammatory gene expression profile using the nCounter Mouse Inflammation Gene Expression CodeSet (Nanostring Technologies, Seattle, USA) according to the manufacturer’s instructions.

Statistics
All experiments were performed at least three times with groups of 3 mice unless otherwise stated. Results are expressed as mean ± SEM. Statistical significance was calculated using ANOVA (*p≤0.05, **p≤0.01, ***p≤0.001).

Results
Ly6C and Ly6G positive myeloid cells accumulate during inflammation and fibrosis in liver and kidney
In order to characterise MDSC arising in vivo after chronic inflammation we induced liver fibrosis via bile-duct ligation (BDL) [29] and kidney fibrosis by feeding mice an adenine rich diet [26]. Furthermore, as a positive control, mice were either injected with LPS/IFNγ, as this has been shown to induce suppressive Gr-1 positive MDSC in the spleen, or in vitro generated BM-derived MDSC were used [2]. Although under steady state conditions, MDSC-like myeloid...
cells are present in the liver, kidney and spleen (Fig. 1A), upon BDL, adenine feeding or LPS/IFNγ treatment, the numbers of CD11b+Gr-1int/high myeloid cells significantly increased in the respective organs (Fig. 1A). We then determined the relative contribution of monocytic myeloid cells (CD11b+Ly6Chigh/Ly6Gneg) and granulocytic myeloid cells (CD11b+Ly6Cint/Ly6Ghigh) within the Gr-1 positive myeloid population (Fig. 1B, C). Both subsets were present in liver and kidney, even under steady-state conditions, but monocytic myeloid cells were significantly increased after BDL whereas granulocytic myeloid cells were enriched in fibrotic kidneys after adenine feeding (Fig. 1B, C). In the spleen of LPS/IFNγ-treated mice and in the bone-marrow cultures both monocytic and granulocytic myeloid cells were present, although the proportion of granulocytic myeloid cells was increased in both cases (Fig. 1B, C). Not only did the proportion of CD11b+Ly6C+ and CD11b+Ly6G+ cells increase in the liver and kidney, respectively, also absolute numbers of both cell types were increased substantially (Fig. 1D, E). Together, these data show that during liver and kidney fibrosis myeloid cells with a different MDSC phenotype accumulate. Furthermore, by separately analysing Ly6G and Ly6C expression, populations of MDSC-like myeloid cells reported to be Gr-1 positive, show differential accumulation depending on the anatomical location.

Monocytic MDSC accumulate in the liver whereas granulocytic MDSC are induced in the kidney during organ inflammation and fibrosis

Suppressive activity has been reported for both monocytic Ly6Chigh expressing and Ly6G expressing myeloid cells [1, 16]. As we found that Ly6G and Ly6C expressing myeloid cells are induced with differential preference in organ fibrosis, cytokine injection in vivo and in vitro culture of bone marrow cells with CSF2 (Fig. 1), we analysed which of these subsets constituted MDSC and thus possessed suppressive capacity. As the development of MDSC is proposed to be IFNγ-dependent and that IFNγ is needed MDSC effector function [12, 13], we sorted CD11b+ myeloid cells according to their expression of Ly6C, Ly6G and IFNγRβ (Fig. 2A) and cocultured them with CFSE-labelled T cells in the presence of anti-CD3ε/CD28 coated beads. Interestingly, IFNγRβ positive Ly6C/G expressing myeloid cells were readily detected in the inflamed spleen and kidney, but not in the chronically inflamed liver after bile duct ligation or the bone marrow cultures. In bile duct ligated mice, Ly6Cpos myeloid cells possessed suppressive function (Fig. 2B), whereas in adenine-fed mice suppressive capacity was present within the renal Ly6Gpos myeloid subset (Fig. 2C). In both the in vivo LPS/IFNγ-induced and bone marrow-derived myeloid cells, suppressive activity was present in both Ly6Gpos and Ly6Cpos subsets (Fig. 2D, E). Moreover, in the kidney suppressive capacity was restricted to IFNγRβ expressing myeloid cells (Fig. 2C). In contrast, in the liver IFNγRβ was not expressed on suppressive myeloid cells (Fig. 2B), nor did the presence or absence of IFNγRβ on splenic myeloid cells influence their suppressive capacity (Fig. 2D). Thus, IFNγRβ expression does not seem to constitute a reliable or unique marker for the identification of functionally active MDSC. Gene expression analysis of a large panel of inflammatory genes revealed that the Ly6Gpos and Ly6Cpos MDSC from liver and kidney are not similarly regulated. Most genes that were differentially expressed by these two subsets, were either part of a Ly6C-signature (also present in Ly6Cpos cells from an adenine-fed kidney), or of a Ly6G-signature in both liver and kidney (S1 Fig.). A subset of genes was specifically regulated in kidney Ly6Gpos MDSC, of which several seem to be involved in the continuous attraction and activation of neutrophillic granulocytes/myeloid cells. Interestingly, neither liver nor kidney MDSC expressed arginase 1 (arg1) or iNOS (nos2) mRNA, indicating that suppressive function in both liver and kidney is not achieved via depletion of arginine or production of ROS. Together, these data indicate that MDSC accumulating during chronic inflammation and fibrosis in liver and kidney can originate from the monocytic
Fig 1. Differential distribution of monocytic and granulocytic myeloid derived suppressor cells within Gr-1 positive cells in liver and kidney inflammation and fibrosis. C57BL/6 mice underwent bile-duct ligation, were fed an adenine-enriched diet, or were injected i.v. with LPS/IFN-γ. Furthermore, BM-MDSC were generated in vitro by culture of bone marrow cells with CSF2 (GM-CSF) (A, B). After the indicated times liver, kidney, spleen or bone marrow cells were isolated and analysed by flow cytometry. Histograms depict

(C) % positive within CD11b+.

(D) # myeloid cells/organ.

(E) # myeloid cells/organ.

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or granulocytic myeloid subset, reflected by their gene-expression profile, depending on the location of induction. Additionally, IFNγRβ expression can be used to identify suppressive MDSC within the Ly6Gpos myeloid compartment in kidney, but not liver, inflammation.

MDSC influence fibrosis progression in both liver and kidney inflammation

Although we find MDSC with potent ex vivo inhibitory function to accumulate during both liver and kidney fibrosis (Fig. 2), it was unclear whether the presence of MDSC would influence fibrosis progression. To test this, we treated bile duct ligated and adenine-fed mice with all-trans-retinoic acid (ATRA) in their drinking water or not, which is known to change MDSC functionality leading to their inability to exert suppressive function ([30]). As a control we sorted CD11b+Gr-1pos cells from the liver of ATRA-treated bile-duct ligated mice and found viable (Hoechst negative), non-parenchymal cells stained with CD11b and Gr-1 (A) or viable, CD11bpos cells stained for Ly6G and Ly6C (B). Representative (A, B) and cumulative (C) data of 4 (liver, spleen) or 3 (kidney, bone marrow) independent experiments are shown (n > 9). Absolute numbers of CD11b+Ly6C+ and CD11b+Ly6G+ myeloid cells in the liver (D) and kidney (E) at the indicated time-points after BDL or adenine-feeding, respectively. Data are depicted as mean +/- SEM. Significance was calculated by ANOVA. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

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![Fig 2. Suppressive capacity of monocytic and granulocytic MDSC subsets.](image)

C57BL/6 mice were treated as in Fig. 1. Gating strategy for sorting (A). At the indicated times myeloid subsets from liver (B), kidney (C), spleen (D) or in vitro bone marrow culture (E) were isolated and CD11b+ cells were sorted on the basis of their Ly6C or Ly6G expression and additional expression of IFNγRβ (A), yielding 4 separate subsets of CD11b+ myeloid cells. Naïve CFSE-labelled CD8 T cells were stimulated using αCD3/αCD28 coated beads and the different subsets of sorted myeloid cells were added at a 3:1 ratio (B-E). After 72h T cell proliferation was analysed by flow cytometry and the percentage of proliferated T cells is depicted (B-E). Cumulative data from 2 independent experiments are shown. Data are depicted as mean +/- SEM. Significance was calculated by ANOVA. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. ND = not detectable.

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that these did not possess suppressive capacity towards T cells in a proliferation assay (data not shown). In both bile-duct ligated and adenine-fed ATRA-treated mice, fibrosis markers like α-SMA, TGF-β, Collagen IV and vimentin were significantly increased (Fig. 3), indicating that the induction of MDSC during chronic inflammation dampens ensuing fibrosis in both the kidney and the liver.

General MDSC specific surface markers not found

So far, one of the major difficulties in the study of MDSC function in inflammation-associated diseases is the absence of a reliable surface marker that is specifically expressed on myeloid cells with suppressive function. Although IFNγRβ expression defined Ly6Gpos MDSC in the setting of kidney fibrosis, we excluded the IFNγRβ as a general marker, since it was not universally expressed on monocytic or granulocytic MDSC (Fig. 4), as it was not expressed on liver-derived Ly6Cpos MDSC. Several other markers have been proposed as markers for MDSC in recent years. Thus, we performed a comprehensive phenotypic analysis to possibly identify a common additional marker for MDSC besides CD11b and Ly6G or Ly6C (Fig. 4 and S2 Fig.). Many of these already proposed markers are indeed expressed on several MDSC populations. For instance, IL-4Rα expression is induced during liver and kidney fibrosis and in the spleen after LPS/IFNγ treatment (Fig. 4). However, in liver and kidney the IL-4Rα is not only expressed on the suppressive subpopulation of myeloid cells, but also on the non-suppressive myeloid subpopulation, indicating that IL-4Rα expression cannot serve as a common marker for suppressive MDSC [33]. Furthermore, B7H1 expressed by MDSC can have a functional role in mediating suppression [17, 31]. In the liver, B7H1 expression was apparently restricted to the Ly6Cpos MDSC subset (Fig. 4). However, in the kidney Ly6Cpos and Ly6Gpos myeloid cells expressed B7H1, excluding B7H1 as a general MDSC specific marker. In order to clarify whether in principle liver and kidney MDSC are functionally similar or not we analysed mRNA expression levels of a panel of inflammation-associated genes (S1 Fig.). These analyses showed that

Fig 3. Fibrosis markers in the liver and kidney after all-trans-retinoic acid treatment. C57BL/6 mice underwent bile-duct ligation, were fed an adenine-enriched diet or as a control were left untreated. After 7 days mice were treated with 1g/L all-trans-retinoic acid (ATRA) in their drinking water (BDL: n = 7, Adenine: n = 4) or not (BDL: n = 8, adenine: n = 4)) for the remaining time until analysis. At day 14 (BDL and adenine feeding), total liver and kidney RNA was isolated for real-time PCR of fibrosis markers. Shown are mRNA expression levels for α-SMA, collagen IV, TGF-β and vimentin relative to the levels in non-treated mice (n = 3), which was set to 1. Data are depicted as mean +/- SEM. Significance was calculated by ANOVA. *p≤0.05, **p≤0.01, ***p≤0.001.

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these expression patterns were organ-independently either Ly6C- or Ly6G-associated but did not associate with suppressive function. Only a small panel of inflammatory genes were exclusively expressed in kidney Ly6Gpos MDSC, known to be involved in the chemotactic attraction of neutrophillic granulocytes (S1 Fig.). However, taken together these data do not identify a universal MDSC marker and supports the notion that such a marker may not exist, although within particular disease entities such additional markers can be identified.

**Discussion**

Currently, the identification of suppressive MDSC in for instance murine tumour models is based on the co-expression of CD11b and Gr-1 on myeloid cells. Such CD11b+Gr-1+ myeloid cells are then tested for their suppressive capacity towards innate or adaptive immune cells to formally prove their MDSC status. Pro-inflammatory molecules such as IL-1β [32], IL-6 [9] and PGE2 [33] are thought to promote the induction of MDSC in tumours, whereas pro-inflammatory TNF signalling can drive the accumulation of MDSC in tumours [34]. Using CD11b and Gr1 surface molecules to identify suppressive myeloid cells, these cells are found in infection [35, 36] and autoimmunity [37]. In this study, we investigated whether in local chronic inflammation MDSC are induced to suppress ongoing inflammatory immune reactions and their resulting organ damage. We now demonstrate, using well-established models of kidney or liver inflammation and fibrosis, that during chronic inflammation, CD11b+Gr1+ myeloid cells expand. Further staining for Ly6C and Ly6G, both of which are recognised by anti-Gr1 antibodies, showed differential expansion of Ly6Gpos and Ly6Cpos myeloid cells. The first preferentially accumulate in the inflamed kidney, whereas the second accumulated significantly in

![Fig 4. Surface marker expression on suppressive and non-suppressive myeloid subsets.](https://doi.org/10.1371/journal.pone.0119662.g004)
the livers of bile duct-ligated mice, not only as percentage within CD11b⁺ myeloid cells but very prominently also in absolute numbers. In the inflamed kidney, suppressive MDSC expressed the IFNγRβ in addition to Ly6G. In contrast, in livers of bile duct ligated mice, suppressive MDSC expressed Ly6C but did not express IFNγRβ. In contrast, BM-derived MDSC did not express IFNγRβ at all, although these cells possessed suppressive capacity. Moreover, although IFNγ was necessary to induce MDSC in the spleen, expression of the IFNγRβ receptor was not required for suppressive activity of these splenic MDSC. The IFNγRα chain binds to IFNγ and the IFNγRβ chain transduces signals to the nucleus [38]. Although the IFNγRα chain is expressed highly on the membranes of B-, T- and myeloid cells, the IFNγRβ is only highly expressed only on the membrane of myeloid cells [39]. We now show that the IFNγRβ is not generally expressed on MDSC, which may explain the contrasting findings reported in literature on the importance of IFNγ for the induction and function of MDSC [10, 12, 13].

In order to classify MDSC induced in a specific organ, we decided to compare them directly to a defined MDSC “standard”, i.e. bone marrow derived and LPS/IFNγ induced splenic MDSC. Surprisingly, compared to BM and splenic MDSC, in the kidney and liver suppressive activity was only present in either Ly6Gpos or Ly6Cpos myeloid cells, respectively. We have recently reported that MDSC can be generated from monocytic cells by activated hepatic stellate cells (HSC) [36], indicating that monocytes arriving in the inflamed liver can locally be differentiated into suppressive MDSC during fibrosis when HSC become activated and differentiate into myofibroblasts. In the kidney such myofibroblasts, important for fibrosis progression, can arise from various subsets of renal cell populations, including fibroblasts, epithelial and endothelial cells [37], indicating that in different organs the cell population and their mechanisms of induction of MDSC may differ considerably.

Extensive phenotypic analysis of all myeloid cell subsets did not reveal a potential candidate for a liver MDSC specific marker. In tumours, IL4Rα expression not only correlates with suppressive function [40], but can also be used to specifically target MDSC [41]. However, in all of the conditions we tested, IL4Rα is expressed on both Ly6Gpos and Ly6Cpos subsets and cannot be used to specifically distinguish suppressive from non-suppressive myeloid subpopulations in kidney or liver inflammation and fibrosis. In livers of bile duct-ligated mice, B7H1 is expressed on the suppressive Ly6Cpos subset. Although in the kidney, spleen and bone marrow such a correlation cannot be detected, we and others have reported that hepatic stellate cells can induce MDSC [42, 43], which repress immune responses via B7H1 [31].

Gene expression analysis of a large panel of inflammatory genes revealed that both Ly6Cpos and Ly6Gpos MDSC were similarly regulated as their non-suppressive counterparts. For instance, Ly6Cpos MDSC in the liver express high levels of CCR2 and also produced the ligand CCL2, similar to for instance Ly6Cpos inflammatory monocytes ([44]). Ly6Gpos MDSC from the kidney did specifically induce the expression of several chemokines and chemokine receptors, involved in the attraction and functional modulation of granulocytes. This may reflect that MDSC in the kidney and liver utilize different signalling modes for further attraction of myeloid cells, which may then also be modulated into becoming MDSC.

Although we could not identify a general marker for MDSC, specific markers for MDSC, like the IFNγRβ in the kidney, may exist in specific disease entities. Thus, our study shows that during chronic inflammation leading to organ fibrosis in the liver and kidney myeloid-derived suppressor cells are efficiently induced capable of inhibiting potentially harmful immune responses.

Supporting Information
S1 Fig. Gene-expression analysis of Ly6C and Ly6G myeloid populations. Sorted cells from single livers and kidneys 14 and 10 days after bile-duct ligation or adenine-feeding, 20,000 cells
were analysed. Average gene-expression of mean-centered data is shown for hepatic CD11b^Ly6C^ (n = 3), CD11b^Ly6G^ (n = 3) and renal CD11b^Ly6C^ (n = 1) and CD11b +Ly6G+ (n = 2) myeloid cells. High and low expression of individual genes is indicated by a colour code.

(TIF)

S2 Fig. Additional phenotypical analysis of MDSC subtypes. Myeloid subsets isolated as in Fig. 1 were stained for various markers described to be associated with MDSC phenotype and/or function. Red squares indicate the suppressive populations.

(TIF)

S3 Fig. Fluorescence minus one (FMO) control staining. FMO for IFNγRβ from a kidney after 10 days of adenine feeding before sorting. Gated on CD11b+Ly6G+ cells within a leukocyte gate without doublets.

(TIF)

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Author Contributions

Conceived and designed the experiments: BH LD ILP. Performed the experiments: BH JM TB CM MW JP LD ILP. Analyzed the data: BH JM TB LD ILP. Contributed reagents/materials/analysis tools: FT PK CK. Wrote the paper: BH LD ILP.

References

1. Movahedi K, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, et al. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. Blood 2008. 111: 4233–4244. doi:10.1182/blood-2007-07-099226 PMID: 18272812
2. Greifenberg V, Ribechini E, Rossner S, Lutz MB. Myeloid-derived suppressor cell activation by combined LPS and IFN-gamma treatment impairs DC development. European journal of immunology 2009. 39: 2865–2876. doi:10.1002/eji.200939486 PMID: 19637228
3. Zhao X, Rong L, Zhao X, Li X, Liu X, et al. TNF signaling drives myeloid-derived suppressor cell accumulation. J Clin Invest 2012. 122: 4094–4104. doi:10.1172/JCI64115 PMID: 23064360
4. Taketo MM. Cyclooxygenase-2 inhibitors in tumorigenesis (Part II). Journal of the National Cancer Institute 1998. 90: 1609–1620. PMID: 9811310
5. Taketo MM. Cyclooxygenase-2 inhibitors in tumorigenesis (part I). Journal of the National Cancer Institute 1998. 90: 1529–1536. PMID: 9790545
6. Cheng P, Corzo CA, Luetette N, Yu B, Nagaraj S, et al. Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. The Journal of experimental medicine 2008. 205: 2235–2249. doi:10.1084/jem.20080132 PMID: 18809714
7. Foell D, Wittkowski H, Vogl T, Roth J. S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. Journal of leukocyte biology 2007. 81: 28–37. PMID: 16943388
8. Bunt SK, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. J Immunol 2006. 176: 284–290. PMID: 16365420
9. Bunt SK, Yang L, Sinha P, Clements VK, Leips J, et al. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. Cancer research 2007. 67: 10019–10026. PMID: 17942936
10. Sinha P, Clements VK, Ostrand-Rosenberg S. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. Journal of immunology 2005. 174: 636–645. PMID: 15634881
11. Sinha P, Clements VK, Bunt SK, Albeida SM, Ostrand-Rosenberg S. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. Journal of immunology 2007. 178: 977–983. PMID: 17617589
12. Kusmartsev S, Gabrilovich DI. Immature myeloid cells and cancer-associated immune suppression. Cancer immunology, immunotherapy 2002. 51: 293–298. PMID: 12111117
13. Mazzoni A, Bronte V, Viartin A, Spitzer JH, Apolloni E, et al. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. Journal of immunology 2002. 168: 689–695. PMID: 11777962
14. Soehnlein O, Lindbom L. Phagocyte partnership during the onset and resolution of inflammation. Nature reviews 2010. 10: 427–439. doi: 10.1038/nri2779 PMID: 20498669
15. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. Journal of immunology, 2009. 182: 4499–4506. doi: 10.4049/jimmunol.0802740 PMID: 19342621
16. Youn Ji, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumour-bearing mice. Journal of immunology 2008. 181: 5791–5802. PMID: 18832739
17. Liu Y, Zeng B, Zhang Z, Zhang Y, Yang R, B7–H1 on myeloid-derived suppressor cells in immune suppression by a mouse model of ovarian cancer. Clinical immunology 2008. 129: 471–481. doi: 10.1016/j.clim.2008.07.030 PMID: 18790673
18. Dietlin TA, Hofman FM, Lund BT, Gilmore W, Stohlman SA, et al. Mycobacteria-induced Gr-1+ subsets from distinct myeloid lineages have opposite effects on T cell expansion. Journal of leukocyte biology 2007. 81: 1205–1212. PMID: 17307863
19. Zhu B, Bando Y, Xiao S, Yang K, Anderson AC, et al. CD11b+Ly-6C(hi) suppressive monocytes in experimental autoimmune encephalomyelitis. Journal of immunology 2007. 179: 5228–5237. PMID: 17911608
20. Luan Y, Mosheir E, Menon MC, Wilson D, Woytovich C, et al. Monocytic myeloid-derived suppressor cells accumulate in renal transplant patients and mediate CD4(+)Foxp3(+) Treg expansion. American journal of transplantation. 13: 3123–3131. doi: 10.1111/ajt.12461 PMID: 24103111
21. Rodriguez PC, Ernstoff MS, Hernandez C, Atkins M, Zabaleta J, et al. Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. Cancer research 2009. 69: 1553–1560. doi: 110.1158/0008-5472.CAN-08-1921 PMID: 19201693
22. Zhang H, Liu Y, Bian Z, Huang S, Han X, et al. The critical role of myeloid-derived suppressor cells and FXR activation in immune-mediated liver injury. Journal of autoimmunity. 53: 55–66. doi: 10.1016/j.jaut.2014.02.010 PMID: 24721598
23. Zeng QL, Yang B, Sun HQ, Feng GH, Jin L, et al. Myeloid-derived suppressor cells are associated with viral persistence and downregulation of TCR zeta chain expression on CD8(+) T cells in chronic hepatitis C patients. Molecules and cells. 37: 66. doi: 10.1038/molcells.2014.2282 PMID: 24552712
24. Hoechst B, Ormandy LA, Ballmaier M, Lehner F, Kruger C, et al. A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4(+)CD25(+)Foxp3(+) T cells. Gastroenterology 2008. 135: 234–243. doi: 10.1053/j.gastro.2008.03.020 PMID: 18485901
25. Hernandez-Gea V, Toffanin S, Friedman SL, Llovet JM. Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. Gastroenterology. 144: 512–527. doi: 10.1053/j.gastro.2013.01.002 PMID: 23313965
26. Tamura M, Aizawa R, Hori M, Ozaki H. Progressive renal dysfunction and macrophage infiltration in interstitial fibrosis in an adenine-induced tubulointerstitial nephritis mouse model. Histochemistry and cell biology 2009. 131: 483–490. doi: 10.1007/s00418-009-0557-5 PMID: 19159945
27. Huang LR, Wohleber D, Reisinger F, Jenne CN, Cheng R., et al. Intrahepatic myeloid-cell aggregates enable local proliferation of CD8(+) T cells and successful immunotherapy against chronic viral liver infection. Nature immunology 2013. 14: 574–583. doi: 10.1038/ni.2573 PMID: 23584070
28. Teteris SA, Hochheiser K, Kurts C. Isolation of functional dendritic cells from murine kidneys for immunologic characterization. Nephrology 2012. 17: 364–371. doi: 10.1111/j.1440-1797.2012.01581.x PMID: 22320441
29. Gujral JS, Liu J, Farhood A, Hinson JA, Jaeschke H. Functional importance of ICAM-1 in the mechanism of neutrophil-induced liver injury in bile duct-ligated mice. American journal of physiology 2004. 286: G499–507. PMID: 14563671
30. Nefedova Y, Fishman M, Sherman S, Wang X, Beg AA, et al. Mechanism of all-trans retinoic acid effect on tumor-associated myeloid-derived suppressor cells. Cancer research 2007. 67: 11021–11028. PMID: 18006848
31. Chou HS, Hsieh CC, Charles R, Wang L, Wagner T, et al. Myeloid-derived suppressor cells protect islet transplants by B7–H1 mediated enhancement of T regulatory cells. Transplantation 2012. 93: 272–282. doi: 10.1097/TP.0b013e31823ff39 PMID: 22179405

32. Song X, Krelin Y, Dvorkin T, Bjorkdahl O, Segal S, et al. CD11b+/Gr-1+ immature myeloid cells mediate suppression of T cells in mice bearing tumors of IL-1beta-secreting cells. Journal of immunology. 2005. 175: 8200–8208. PMID: 16339559

33. Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. Cancer research 2007. 67: 4507–4513. PMID: 17483367

34. Zhao X, Rong L, Zhao X, Li X, Liu X, et al. TNF signaling drives myeloid-derived suppressor cell accumulation. The Journal of clinical investigation 2012. 122: 4094–4104. doi: 10.1172/JCI64115 PMID: 23064360

35. Sunderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, et al. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. Journal of immunology. 2004. 172: 4410–4417. PMID: 15034056

36. Voisin MB, Buzoni-Gatel D, Bout D, Velge-Roussel F. Both expansion of regulatory GR1+ CD11b+ myeloid cells and anergy of T lymphocytes participate in hyproresponsiveness of the lung-associated immune system during acute toxoplasmosis. Infection and immunity 2004. 72: 5487–5492. PMID: 15322051

37. Haile LA, von Wasselewski R, Garmrekelashvili J, Kruger C, Bachmann O, et al. Myeloid-derived suppressor cells in inflammatory bowel disease: a new immunoregulatory pathway. Gastroenterology 2008. 135: 871–881, 881 e871–875. doi: 10.1053/j.gastro.2008.06.032 PMID: 18674538

38. Pestka S, Kotenko SV, Muthukumarana G, Izotova LS, Cook JR, et al. The interferon gamma (IFN-gamma) receptor: a paradigm for the multichain cytokine receptor. Cytokine & growth factor reviews 1997. 8: 189–206.

39. Bernabei P, Coccia EM, Rigamonti L, Bosticardo M, Forni G, et al. Interferon-gamma receptor 2 expression as the deciding factor in human T, B, and myeloid cell proliferation or death. Journal of leukocyte biology 2001. 70: 950–960. PMID: 11739558

40. Mandruzzato S, Solito S, Falisi E, Francescato S, Chiarion-Sileni, et al. IL4Ralpha+ myeloid-derived suppressor cell expansion in cancer patients. Journal of immunology. 2009. 182: 6562–6568. doi: 10.4049/jimmunol.0803831 PMID: 19414811

41. Roth F, De La Fuente AC, Vella JL, Zoso A, Inverardi L, et al. Aptamer-mediated blockade of IL4Ralpha triggers apoptosis of MDSCs and limits tumor progression. Cancer research 2012. 72: 1373–1383. doi: 10.1158/0008-5472.CAN-11-2772 PMID: 22282665

42. Chou HS, Hsieh CC, Yang HR, Wang L, Arakawa Y, et al. Hepatic stellate cells regulate immune response by way of induction of myeloid suppressor cells in mice. Hepatology 2011. 53: 1007–1019. doi: 10.1002/hep.24162 PMID: 21374665

43. Hochst B, Schildberg FA, Sauernborn P, Gabel YA, Gevensleben, et al. Activated human hepatic stellate cells induce myeloid derived suppressor cells from peripheral blood monocytes in a CD44-dependent fashion. Journal of hepatology 2013. 59: 528–535. doi: 10.1016/j.jhep.2013.04.033 PMID: 23665041

44. Lauvau G, Chorro L, Spaulding E, Soudja SM. Inflammatory monocyte effector mechanisms. Cellular immunology. 2014. 291: 32–40. doi: 10.1016/j.cellimm.2014.07.007 PMID: 25205002