Generation and functional characterization of MDSC-like cells

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ABSTRACT

Myeloid-derived suppressor cells (MDSC) are critical in regulating immune responses by suppressing antigen presenting cells (APC) and T cells. We previously observed that incubation of peripheral blood monocytes with interleukin (IL)-10 during their differentiation to monocyte-derived dendritic cells (moDCs) results in the generation of an APC population with a CD14+HLA-DRlow phenotype (IL-10-APC) with reduced stimulatory capacity similar to human MDSC. Co-incubation experiments now revealed that the addition of IL-10-APC to moDC caused a reduction of DC-induced T-cell proliferation, of the expression of maturation markers, and of secreted cytokines and chemokines such as TNF-α, IL-6, MIP-1α and Rantes. Addition of IL-10-APC increased the immunosuppressive molecule osteoactivin and its corresponding receptor syndecan-4 on moDC. Moreover, CD14+HLA-DRlow MDSC isolated from healthy donors expressed high levels of osteoactivin, which was even further upregulated by the auxiliary addition of IL-10. Using transcriptome analysis, we identified a set of molecules and pathways mediating these effects. In addition, we found that IL-10-APC as well as human isolated MDSC expressed higher levels of programmed death (PD)-1, PD-ligand-1 (PD-L1), glucocorticoid-induced-tumor-necrosis-factor-receptor-related-protein (GITR) and GITR-ligand. Inhibition of osteoactivin, syndecan-4, PD-1 or PD-L1 on MDSC by using blocking antibodies restored the stimulatory capacity of DC in co-incubation experiments. Activation of MDSC with Dectin-1 ligand curdlan reduced the expression of osteoactivin and PD-L1. Our results demonstrate that osteoactivin syndecan-4 and PD/-PD-L1 are key molecules that are profoundly involved in the inhibitory effects of MDSC on DC function and might be promising tools for clinical application.

Abbreviations: APC, antigen presenting cells; CENA, co-expression network analysis; DC, dendritic cells; DE genes, differentially expressed genes; GITR, glucocorticoid-induced-tumor-necrosis-factor-receptor-related-protein; GITRL, ligand of GITR; GM-CSF, granulocyte-macrophage colony-stimulating factor; GO, gene ontology; GvHD, graft versus host disease; IL-10, interleukin 10; IL-10-APC, APC population with a CD14+HLA-DRlow phenotype; IL-4, interleukin 4; IL-6, interleukin 6; LPS, lipopolysaccharide; MDSC, myeloid-derived suppressor cells; MIP-1α, macrophage inflammatory protein 1α (CCL3); MLR, mixed lymphocyte reaction; moDC, monocyte-derived dendritic cells; PCA, principal component analysis; PD-1, programmed death 1; PD-L1, programmed death ligand 1; RNA-Seq, RNA-sequencing; Th, T helper; TLR, toll-like receptor; TNF-α, tumor necrosis factor α

Introduction

One of the immune system’s most essential roles is to keep the balance between the defense against microbial pathogens or malignant cells and the simultaneous limitation of auto-aggressive immune responses.

Dendritic cells (DC) are the most powerful antigen presenting cells (APC) orchestrating adaptive immune responses. The presence of a specific antigen as well as the appropriate expression of co-stimulatory molecules on APC is necessary for the efficient activation and induction of CD8+ T-cell responses. Myeloid-derived suppressor cells (MDSC) are critical in the regulation of such immune responses by suppressing the function of APC and T cells. They represent a very heterogeneous population of immature myeloid cells whose origin, exact classification and development is still not well understood. In mice, MDSC are usually defined as either CD11b+Ly-6GlowLy-6Clow (Gr-1high) or CD11b+Ly-6G+Ly-6Chigh (Gr-1low), whereas in humans MDSC generally express CD33. The problem of a uniform classification is among others that different human tumors harbor different MDSC subsets. These can be divided into three groups, such as LIN+HLA-DR−low, CD14+HLA-DR−low and CD15+HLA-DR−low. MDSC expand during chronic inflammatory conditions and significantly influence tumor-associated immunosuppression, thereby...
promoting tumor progression and affecting antitumor therapies.5-7

Meanwhile, MDSC can be useful in situations where immunosuppression is warranted, such as some auto-immune diseases or graft-versus-host-disease (GvHD). Consequently, numerous attempts have been made to modulate the number or the immunosuppressive potential of MDSC.

We and others have shown before that interleukin-10 (IL-10) treatment of monocytes during their differentiation process to monocyte-derived DC (moDC) generates a CD14+HLA-DRlow cell subset (IL-10-APC) that resembles human MDSC.9,10

In general, IL-10 is an anti-inflammatory cytokine that regulates T helper (Th) cell responses by inhibiting Th1 activity. It exerts diverse, pleiotropic effects in immunoregulation and inflammation and is primarily produced by monocytes, but also by Th2 lymphocytes, regulatory T cells, mast cells and in certain subsets of activated T and B cells.

It has been demonstrated that IL-10 blocks DC function by inhibiting cytokine production, expression of MHC class II and co-stimulatory molecules and causes a reduced capacity to stimulate the DC-induced proliferation of T cells.8,10-12 Furthermore, IL-10 treatment of DC causes upregulation of the IL-10 receptor (IL-10R), indicating that IL-10 mediates its effects by autoregulation via a negative feedback loop.

When investigating intracellular signaling cascades, we previously found the protein expression of MyD88, TRIF, IRAK1 and TRAF6 to be blocked and the nuclear translocation of NF-κB members RelB and c-Rel as well as interferon-regulatory factor (IRF)-8 and IRF-3 to be inhibited by IL-10. Interestingly, we could show that the mRNA transcription of genes remained unaffected indicating that IL-10 treatment results in a posttranslational degradation of the adaptor molecules.9 In our present study, we show that APC generated in vitro under DC-driving conditions in the presence of IL-10 have the phenotype and function of MDSC and display relevant cell intrinsic differences. Furthermore, we demonstrate that the interaction with osteoactivin/syndecan-4 is involved in the inhibitory effects of these cells.

Results

IL-10 leads to the differentiation of monocytes into MDSC-like cells under DC-driving conditions

Monocytes were differentiated into moDC within 7 d using IL-4 and GM-CSF (Fig. 1A). These moDC express CD1a to a high extent and upregulate the DC activation markers CD80, CD83 and CD86 upon TLR stimulation (Fig. 1B and C, upper two rows). Additional incubation of monocytes with IL-10 during the differentiation into moDC resulted in the generation of an APC population with a CD14+HLA-DRlow phenotype (IL-10-APC; Fig. 1B, third row) similar to human MDSC. Of note, IL-10-APC expressed markedly less CD1a, the bona fide DC marker and rather had the morphologic features of monocytes. Furthermore, upregulation of CD80, CD83 and CD86 on IL-10-APC upon stimulation with TLR-4 ligand LPS was relevantly diminished in contrast to moDC (Fig. 1C).

Co-incubation of IL-10-APC with moDC results in reduced capacity to stimulate allogeneic T cells

In the next set of experiments, we analyzed whether IL-10-APC not only phenotypically resemble human MDSC, but also have T-cell suppressive potential. Therefore, we incubated TLR-activated or naïve moDC with monocytes as a control or IL-10-APC together with allogeneic T cells. Whereas allogeneic T cells cultured with LPS-activated moDC alone or with further addition of monocytes showed high proliferation in an in vitro mixed lymphocyte reaction assay, co-incubation with IL-10-APC resulted in a profound reduction of T-cell proliferation that almost reached the extent of T cells cultured in the complete absence of stimulatory DC (Fig. 1D). This indicates that IL-10-APC profoundly abrogated the stimulation of T lymphocytes. Of note, these effects were not due to induction of IL-10 production and were not observed when purified CD14+ monocytes were used as a control in the experiments. Furthermore, this effect was dose-dependent, as higher numbers of IL-10-APC suppressed DC-mediated T-cell proliferation to a greater extent (Fig. 1E).

IL-10-APC have a direct effect on the phenotype and function of moDC

We next questioned whether IL-10-APC can directly influence the phenotype of moDC and their further stimulation with LPS by incubating naive moDC with or without IL-10-APC. Staining of DC surface markers indeed revealed a marked downregulation of CD1a, CD80, CD83 and CD86 upon TLR stimulation in the presence of IL-10-APC (Fig. 1C, fourth and fifth rows) and a re-induction of CD14 expression (Fig. 1B, fourth and fifth rows).

When supernatants out of these co-cultures were analyzed in regard to cytokine and chemokine production, we found that the addition of IL-10-APC to the culture resulted in significantly decreased amounts of the secreted chemokines MIP1-α (CCL3) and RANTES (CCL5) (Fig. 1F and G), the pro-inflammatory cytokines TNF-α and IL-6 (Fig. 1I and J) and the immunosuppressive IL-10 (Fig. 1H). Taken together, these experiments revealed that IL-10-APC are not only immunosuppressive themselves, but also have a direct, profound impact on DC phenotype and function as demonstrated by decreased upregulation of DC activation markers and diminished cytokine production.

IL-10-APC display relevant cell intrinsic differences compared to moDC

We hypothesized that the observed differences between moDC and IL-10-APC might be explained by treatment-specific activation of distinct molecular pathways. To assess transcriptional changes on a global scale, we performed whole transcriptome analysis using quantitative RNA-sequencing (RNA-Seq) and compared the transcriptomes of moDC and IL-10-APC in immature or maturated state. To do so, we generated moDC and IL-10-APC in the presence of GM-CSF/IL-4 with or without IL-10, respectively, and induced maturation by treatment with LPS. To ensure sample quality, we checked the CD1a and
CD83 expression as well as the migratory capacity of the cells and thereby confirmed all four different cell populations to be present before RNA-Seq. After preprocessing, normalization and quality control of the RNA-Seq data, the variance of transcription within the complete data set was visualized using unbiased principal component analysis (PCA) based on all 13,108 present genes (Fig. 2A and B). Additionally, hierarchical clustering (HC) of the top 1,000 variable genes \( p < 0.05 \) was performed (Fig. 2C). These analyses showed that both IL-10 and LPS treatment induced distinct transcriptional profiles that divided the cells into three definite clusters. The visualized expression values depict strong differences between immature...
and LPS-treated moDC as well as between moDC and IL-10-APC. In contrast, the IL-10-APC show a high degree of similarity. LPS treatment did not elicit major transcriptional changes in these cells. The gene set induced by LPS in moDC is mostly unaffected by LPS-treatment in IL-10-APC. We further quantified the global distribution of variance within the data set by comparing the numbers of significantly differentially expressed (DE) genes (fold change (FC) > 2 or < −2, \( p < 0.05 \)) (Fig. S1A), which further demonstrated the cellular differences between moDC and IL-10-APC and illustrate the diverging responses to LPS.

To investigate the decisive information within the data set in more detail, we focused our subsequent analysis on the union of DE genes from the following two comparisons: immature IL-10-APC versus immature moDC and LPS-treated IL-10-APC versus LPS-treated moDC (Fig. S1A). These 3,504 genes describe the critical variance within the data set and encompass the relevant information on cellular and LPS response-specific
differences. To elucidate the expression of these genes across the cell types and treatments, we performed a co-expression network analysis (CENA) and colored the genes according to their condition-specific expression (Fig. 2D). The network analysis clustered the genes based on the similarity of their expression pattern and thereby enabled the identification of gene sets specifically regulated in each condition. Importantly, the network reflects the previously described tripartition of the samples. The central cluster consists of genes that are specifically upregulated in immature moDC, whereas the distant cluster at the upper margin comprises genes that are upregulated due to LPS treatment in moDC. The broader cluster in the lower part of the network is composed of genes specifically upregulated under the influence of IL-10. It describes the phenotype of IL-10-APC both with and without LPS treatment. To identify enriched functions of the distinct gene sets, we performed gene ontology enrichment analysis on the upregulated genes (Group FC>1) of the respective conditions (Table S1: Gene Ontology Enrichment Analysis (GOEA) of co-expression analysis). We found that genes specifically upregulated in immature moDC are connected to basic cellular processes like adhesion and localization, cellular development as well as metabolic processes. As expected, LPS treatment of moDC induced the expression of genes that are connected to an immunological response of the cells. We found GO terms like "innate immune response," "defense response" as well as "response to interferon-gamma" to be significantly enriched for these genes. The application of IL-10 during the process of differentiation induced the expression of genes also involved in the activation of immunological functions. Significantly enriched GO terms for this gene set comprise the terms "regulation of immune system process," "regulation of leukocyte activation," "response to stimulus" and "immune response-activating signal transduction." As mentioned above, LPS-treatment of IL-10-APC did not elicit major transcriptional changes. Consequently, the respective enriched GO terms show a very high overlap. However, GO terms uniquely enriched in LPS-treated IL-10-APC included "response to lipopolysaccharide" and "toll-like receptor signaling" pathway. Taken together, this indicates that the IL-10-APC maintain their IL-10-induced characteristics upon LPS-treatment even though they are able to sense the immunological stimulus.

To determine key genes potentially critical for the observed phenotypes, we further investigated the genes from the above mentioned comparisons with regard to their fold changes (Fig. 2E). As the immunological features of the cells were our main interest, we first determined all genes connected to the GO term "immune system process" within the 3,504 genes (black outline). These 729 genes form the basis for the subsequent analyses. Additionally, we defined genes within two areas of the plot as particularly relevant for our analysis. The area in the lower middle section, labeled in yellow, contains genes that are specifically upregulated upon LPS treatment in moDC but remain unaffected by LPS treatment in IL-10-APC (Fig. S2). Using GOEA, we could identify numerous genes connected to the GO terms "inflammatory response," "positive regulation of lymphocyte proliferation" and "cellular response to lipopolysaccharide" within this area (Table S2: GOEA for selected areas of the ratio-plot). The area in the upper right, highlighted in green, encompasses genes crucial for the IL-10-induced phenotype. These genes are upregulated due to IL-10 independent of LPS (Fig. S3). Since we detected immunoregulatory functionality for the IL-10-APC in previous experiments, we aimed to identify corresponding genes in this area of the plot. GOEA revealed numerous genes linked to the GO terms "negative regulation of immune system process," "negative regulation of response to stimulus" and "negative regulation of T-cell activation."

Based on the above indicated GO terms and additional literature mining, we eventually determined a set of genes that hold the potential to explain the observed phenotypes. We examined the expression of these genes across all samples and grouped them according to their functionality (Fig. 2F). The expression of classical maturation markers including CD40 and CD80 (cluster c1) clearly demonstrates that IL-10-APC do not mature despite treatment with LPS. While moDC showed marked upregulation of these molecules upon treatment with LPS, their expression was completely unaffected in IL-10-APC. Furthermore, we identified additional genes associated with the activation state of DC (c2). High CD14 expression serves as an indicator for the retention of the monocytic phenotype of IL-10-APC. Importantly, we found TSC22D3, also known as GILZ, among the genes which were specifically upregulated in IL-10-APC. This transcription factor is described to be a critical factor in macrophage endotoxin tolerance and could explain the absent response of IL-10-APC to LPS. The immunoregulatory actions of TSC22D3 are considered to be mediated, in part, via the inhibition of NF-κB and AP-1. The increase in expression of the subunits of AP-1, FOS/FOSB and JUN, in IL-10-APC might thus be counteracted by TSC22D3. Additionally, we found TMEM176A, TMEM176B, SPRY1 as well as MAFB and IRAK3 to be upregulated in IL-10-APC, which are associated with an immature state of DC. For example, the role of TMEM176A and TMEM176B to restrain DC maturation has been demonstrated by expression alteration experiments and SPRY1 has been described to hamper monocyte maturation by interfering with IFN-inducible signaling pathways. Furthermore, we found the transcription factor TFCP2L1 and members of the Kruppel-like transcription factor family to be upregulated in IL-10-APCs, which are also described to play critical roles in monocyte differentiation.

Corresponding to the immunomodulatory phenotype of IL-10-APC, we could identify a variety of genes that are known to convey an immunoinhibitory effector function (c3). Besides IL-10, syndecan-4 and osteoactivin, we also found TIGIT, CD55, CD59, S100A9, CD163, HMOX-1 and several other immunoinhibitory molecules to be upregulated in IL-10-APC. Whereas PD-L1 expression was only upregulated in LPS-treated moDC on RNA level, its parologue PDCD1LG2 showed high expression in IL-10-APC. Interestingly, we found that high expression of Dectin-1 (CLEC7A) is stable in IL-10-APC, but downregulated in moDC upon LPS treatment.

To further illustrate the missing response to LPS in IL-10-APC, we depicted the expression of selected classical LPS-response molecules, including members of the STAT family, RELA, RELB and NF-κB1 as well as IRF1 and IRF4 (c4).

In conclusion, the transcriptome analysis revealed a two-part model for the effects of IL-10 on differentiating monocytes.
On the one hand, IL-10-APC maintain an immature state and are insensitive toward LPS. On the other hand, the cells exhibit an immunoinhibitory phenotype (Fig. 2G).

**IL-10-APC express immunosuppressive molecules on their surface and induce them on moDC upon co-culture**

To further investigate the possible mechanisms involved in the inhibitory effects of IL-10-APC in more detail, we analyzed the expression of selected molecules that were found to be differentially regulated by IL-10 in our transcription assays or that have been reported to affect DC and T-cell function.

Osteoactivin, syndecan-4 as well as glucocorticoid-induced tumor-necrosis-factor-receptor-related-protein (GITR), its ligand GITRL, programmed death (PD)1 and its ligand PD-L1 have all been associated with immunosuppressive properties. Using FACS analysis, we found that IL-10-APC in contrast to moDC express higher levels of GITR, GITRL, syndecan-4 and PD-1. Furthermore, we were able to confirm our results obtained from the transcriptome analysis by showing that IL-10-APC express higher levels of osteoactivin, whereas PD-L1 was not upregulated (Fig. 3A, first three rows). Interestingly, upon co-incubation of moDC together with IL-10-APC, the expression of GITR, PD-1, PD-L1 and osteoactivin on immature moDC was markedly upregulated, whereas on mature moDC only GITR and PD-1 were increased (Fig. 3A, last two rows). These co-incubation experiments revealed that IL-10-APC not only express inhibitory molecules themselves, but also induce the induction of immunosuppressive molecules on moDC, thereby exerting a direct immunosuppressive effect on other cells in close proximity.

**Blockade of osteoactivin, syndecan-4, PD-L1 or PD-1 restored the proliferation of T cells incubated with IL-10-APC**

To analyze the possible role of these molecules in transferring inhibitory effects, IL-10-APC and moDC were incubated together with blocking antibodies specific for osteoactivin, syndecan-4, PD-L1, PD-1 and then applied in an MLR assay. In contrast to the addition of the control antibody anti-human IgG, all other antibodies restored the proliferation of T cells in this assay (Fig. 3B), remarkably, in comparison to LPS-activated moDC co-cultured with IL-10-APC. The effect was most pronounced when antibodies against syndecan-4 and osteoactivin were combined (Fig. 3B), indicating that both molecules might act in an additive manner.

**Circulating blood MDSC upregulate inhibitory molecules upon IL-10 exposure**

All previous experiments have been performed with moDC. However, we were interested whether circulating human MDSC show similar effects, thereby mimicking a real clinical situation and strengthening our data. We therefore isolated CD14⁺HLA-DRlow MDSC out ofuffy coats from healthy donors and treated these cells with IL-10. In line with our results obtained from monocyte-derived, GM-CSF/IL-4 generated IL-10-APC, we found a higher expression of GITR, GITRL, syndecan-4, osteoactivin and PD-L1 on MDSC treated with IL-10 in contrast to untreated circulating human MDSC (Fig. 3C). Taken together, this indicates that the observed effects not only apply for IL-10 treated moDC, but are also relevant for human circulating MDSC.

**β-Glucans can influence the function of MDSC**

We found that genes of the C-type lectin/C-type lectin-like domain superfamily are upregulated in IL-10 treated moDC in our transcriptome analysis. Using FACS-analysis, we
confirmed that human Dectin-1, a C-type lectin that was shown to be the major receptor for fungal β-glucans, was markedly upregulated on monocyte-derived IL-10-APC in contrast to moDC (Fig. 4A showing the MFI, as well as percentages, 98% of IL-10-APC and 67% of moDC, data not shown). In line with these results, Dectin-1 was also upregulated on circulating CD14<sup>+</sup>HLA-DR<sup>low</sup> MDSC treated with IL-10 (Fig. 4B).

It has been described previously that activation of β-glucans can subvert the suppression of MDSC, e.g., by inducing MDSC apoptosis and monocytic MDSC differentiation to APC. Consequently, we analyzed the effects of curdlan on CD14<sup>+</sup>HLA-DR<sup>low</sup> cells with MDSC phenotype isolated out of buffy coats from healthy donors. We found that incubation with curdlan for 24 h resulted in a significant downregulation of the percentage of the immunosuppressive molecules PD-1, PD-L1, GITR, GITRL (Fig. 4C), as well as osteoactivin and, however much less pronounced, syndecan-4 (Fig. 4D) on the surface of CD14<sup>+</sup>HLA-DR<sup>low</sup> MDSC. In contrast, incubation of MDSC with TLR 2, 3, 4 and 7/8 ligands upregulated or did not significantly affect the expression of these molecules on the cell surface (Fig. S4A–C), thereby making an unspecific effect of curdlan unlikely.

Figure 4. The β-Glucan Curdlan subverts the function of MDSC. The immunosuppressive molecule dectin-1 was analyzed on the surface of moDC and IL-10-APC (A) as well as on blood CD14<sup>+</sup> HLA-DR<sup>low</sup> MDSC of healthy donors before and 24 h after co-culture with IL-10 (B). Blood CD14<sup>+</sup> HLA-DR<sup>low</sup> MDSC of healthy donors were treated for 24 h with Curdlan and were then analyzed for expression of GITR, GITRL, PD-1, PD-L1 (C), osteoactivin and syndecan-4 (D). Results are from one experiment representative of least three. The significance was calculated according to one-way ANOVA Dunnett’s Multiple Comparison Test and is related to the PBMC control. "p < 0.01.
Discussion

MDSC play an important role in the regulation of immune responses by suppressing the function of APC and T cells. Modulating MDSC function and frequencies would be an optimal target to dictate the success of antitumor therapies or treat autoimmune diseases. Yet, this remains difficult since MDSC generation in sufficient numbers is still a complex process and not much is known about the mechanisms mediating the inhibitory effects of MDSC.

We found that the incubation of human monocytes during their differentiation to moDC with IL-10 in the presence of the cytokines IL-4 and GM-CSF leads to the generation of an APC population that expresses CD14⁺ and HLA-DRlow (IL-10-APC), similar to a human MDSC subpopulation. These IL-10-APC also expressed less co-stimulatory molecules, produced fewer pro-inflammatory cytokines and showed a markedly reduced T-cell stimulatory capacity.

Of note, co-incubation experiments revealed that IL-10-APC are not only suppressive themselves, but also influence surrounding immune cells. The addition of IL-10-APC to moDC resulted in an inhibition of DC-induced T-cell proliferation, a reduced expression of the DC marker CD1a as well as a diminished expression of the maturation markers CD83, CD80, CD86 upon TLR-ligation. Accordingly, IL-10-APC not only phenotypically resemble human MDSC, but also exert immunosuppressive functions dose dependently. Most notable, IL-10-APC would allow a more feasible approach to generate sufficient numbers of MDSC as treatment option by isolating monocytes out of peripheral blood of patients or donors and culturing them with our described cytokine cocktail.

At the same time this indicates that a combination of soluble factors, usually produced locally at the site of an inflammatory process, determines the phenotype and function of the induced APC population. This emphasizes a potential plasticity of monocytes and APC,²⁹,³⁰ and deepens the question what exactly causes this re-programming.

To further analyze possible mechanisms involved in the inhibitory effects of IL-10-APC, we conducted comparative transcriptionomics of moDC and IL-10-APC. We found that gene clusters associated with the regulation of inflammatory and immune responses differed markedly. IL-10-APC maintained an immature state and were insensitive toward LPS-treatment. In addition, IL-10-APC in contrast to moDC showed an upregulation of immunosuppressive markers such as S100A9, osteoactivin, syndecan-4 and genes of the C-type lectin/C-type lectin-like domain superfamily including Dectin (CLEC7A).

Phenotypical characterization using FACS confirmed an increased expression of osteoactivin and its corresponding receptor syndecan-4 on IL-10-APC, as well as an upregulation of PD-1, PD-L1, GITR and GITRL. Interestingly, upon co-incubation of moDC with IL-10-APC, expression of osteoactivin, syndecan-4, GITR as well as PD-1 was increased on moDC, demonstrating that not only IL-10-APC themselves express these inhibitory molecules, but also induce their induction on moDC in co-incubation experiments, thereby transferring a direct immunosuppressive effect on other cells. In summary, our findings argued for substantial phenotypical and functional differences after treatment with IL-10.

We next aimed to exploit the described molecules that have been suggested to affect the immunosuppressive potential of MDSC and DC. Recently, it was described that the transmembrane glycoprotein osteoactivin (DC-HIL) represents such a negative regulator of T-cell activation. Its inhibitory effects are mediated through binding of osteoactivin on DC to its ligand syndecan-4 on T cells.¹⁹,²¹,²³ So far, syndecan-4 was shown to be expressed only on a subset of effector/memory T cells. It has no direct physical interaction with the TCR, but supresses T-cell activation by stimulating CD148 via its protein tyrosine phosphatase activity.¹⁸,²⁰ Furthermore, syndecan-4 mediates its suppressive function independent of regulatory T cells.¹⁸,²⁰,²¹ Osteoactivin differs from other inhibitory molecules since it is not only expressed on lymphoid, but also on non-lymphoid cells such as melanocytes, keratinocytes and osteoblasts and has an intracellular ITAM.³¹-³³ Cross-linking of the osteoactivin receptor with specific antibodies was shown to induce tyrosine phosphorylation of the ITAM,²⁰,²² suggesting that activation of osteoactivin induces MDSC to express IFNγ, nitric oxide and reactive oxygen species.²⁰ Mice lacking the osteoactivin/syndecan-4 pathway are characterized through a massive expansion of pathogenic T cells during experimental autoimmune encephalomyelitis²⁰ or following allogeneic bone marrow transplantation, an effect that might be mediated through a combination of hyperactive APC and disabled MDSC.²¹ We have described previously that osteoactivin is upregulated on DC upon stimulation with IL-10 or other small molecules including the tyrosine kinase inhibitor imatinib, whereas blocking osteoactivin increases the stimulatory capacity of DC.⁹,³⁴

The interaction of GITR/GITRL was shown to have stimulatory effects on T-cell-mediated immune responses in mice. However, its role in DC biology is not completely understood and there seem to be differences between its effects in mice and humans. Baltz et al. recently found that signaling via GITRL downregulates the expression of the immunostimulatory molecules CD40 and CD54 as well as the adhesion molecule EpCAM. Furthermore, GITRL induced production of the immunosuppressive cytokine TGF-β by tumor cells. Blocking GITR-GITRL interaction led to a substantially increased NK cell-mediated cytotoxicity and IFN-γ production of NK cells. GITRL-Ig fusion protein or cell surface-expressed GITRL reduced the nuclear localized c-Rel and RelB. These results indicate that GITR/GITRL interaction can inhibit the function of immune cells in humans.¹⁶,¹⁷

Finally, PD-1/PD-L1 are two molecules that are highly involved in transferring immunosuppression. PD-1 represents an immune checkpoint that regulates the immune system by preventing the activation of T-cells, which in turn reduces autoimmunity and promotes self-tolerance.³⁵ Multiple antibodies against PD-1 and PD-L1 have been developed and are already successfully applied in the clinics.

Remarkably, inhibition of these inhibitory molecules by using specific blocking antibodies against osteoactivin, syndecan-4, PD-L1 or PD-1 restored the function of DC in co-incubation experiments. The option of blocking specific molecules
with antibodies to dampen the immunosuppressive power of MDSC is extremely promising for clinical-translational approaches. Of note, a combination of two antibodies, in our case against osteoactivin and syndecan-4, unleashed an even more pronounced effect, suggesting that additive mechanisms might apply.

For characterization of further molecular mechanisms involved in the differentiation and function of IL-10-APC, we additionally determined the influence of Dectin-1 activation on the immunosuppressive function of IL-10-APC. It was recently demonstrated that glucans can reverse the inhibitory function of monocytic MDSC in mice. Curdlan is a linear $\beta$-1,3 glucan and a Dectin-1 agonist. To determine the role of Dectin-1 activation in human MDSC, IL-10-APC were incubated with curdlan and the phenotype was analyzed. We found that the addition of curdlan, but not of the TLR ligands 2/3/4 or 7/8 resulted in a significant reduction of the number of cells positive for the immunosuppressive molecules PD-1, PD-L1, GITR, GITRL as well as osteoactivin on the surface of IL-10-APC and CD14$^+$HLA-DRlow MDSC. This suggests that curdlan indeed subverts the immunosuppressive potential of MDSC in our experimental setting by downregulation of immunosuppressive molecules.

Last, we wanted to show that our described mechanism is not restricted to IL-10-APC, but also applies for purified, circulating human MDSC. To accomplish this, we isolated CD14$^+$HLA-DRlow MDSC out ofuffy coats from healthy donors and treated these cells with IL-10. In line with our results obtained from monocyte-derived, GM-CSF/IL-4-generated IL-10-APC, we found a higher expression of GITR, GITRL, syndecan-4, osteoactivin and PD-L1 on purified MDSC treated with IL-10 in contrast to untreated circulating human MDSC. Furthermore, human Dectin-1 was also upregulated on purified MDSC. This indicates that the described immunosuppressive molecules might also be upregulated in vivo whenever the cells enter an environment where distinct soluble factors—such as IL-10—are present, e.g., at sites of a chronic inflammatory process.

In summary, our results demonstrate that osteoactivin/syndecan-4 and PD-1/PD-L1 are key molecules that are profoundly involved in the regulation of the inhibitory effects of MDSC on DC function. Furthermore, these cells can be efficiently generated in vitro and might be promising tools for an application in clinical settings where immunosuppression is warranted such as GvHD or other autoimmune disorders.

**Methods**

**Generation of moDC and IL-10-APC**

Buffy coats for human monocyte isolation were obtained from healthy donors and treated with IL-10. In line with our results obtained from monocyte-derived, GM-CSF/IL-4-generated IL-10-APC, we found a higher expression of GITR, GITRL, syndecan-4, osteoactivin and PD-L1 on purified MDSC treated with IL-10 in contrast to untreated circulating human MDSC. Furthermore, human Dectin-1 was also upregulated on purified MDSC. This indicates that the described immunosuppressive molecules might also be upregulated in vivo whenever the cells enter an environment where distinct soluble factors—such as IL-10—are present, e.g., at sites of a chronic inflammatory process.

In summary, our results demonstrate that osteoactivin/syndecan-4 and PD-1/PD-L1 are key molecules that are profoundly involved in the regulation of the inhibitory effects of MDSC on DC function. Furthermore, these cells can be efficiently generated in vitro and might be promising tools for an application in clinical settings where immunosuppression is warranted such as GvHD or other autoimmune disorders.
Determination of chemokine and cytokine production

Secretion of chemokines (CCL3 and CCCL5) and cytokines (TNF-α, IL-6 and IL-10) in co-culture supernatants was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) from R&D Systems, following the manufacturer’s instructions. The readout was performed using a Synergy2 microplate reader (BioTek).

Statistical analysis

All experiments were performed at least 3–4 times, with only representative experiments shown. To analyze statistical significance, a Student’s t-test was used. Comparisons were made as indicated with the Mann–Whitney test or one way analysis of variance (ANOVA) and Dunnett’s using Prism 5 software (Graphpad Software).

RNA isolation

For RNA isolation 5 × 10^6–2 × 10^7 cells per sample were harvested, subsequently lysed in TRIzol (Invitrogen), and total RNA was extracted with the miRNeasy kit (Qiagen) according to the manufacturer’s protocol. The quality of the RNA was assessed by measuring the ratio of absorbance at 260 nm and 280 nm using a Nanodrop 2000 Spectrometer (Thermo Scientific) as well as by visualization of the integrity of the 28S and 18S bands via a RNA analysis ScreenTape assay on a 2200 TapeStation instrument (Agilent).

Generation of cDNA libraries

Total RNA was converted into libraries of double stranded cDNA molecules as a template for high throughput sequencing following the manufacturer’s recommendations using the Illumina TruSeq RNA Sample Preparation Kit v2. Shortly, mRNA was purified from 100 ng of total RNA using poly-T oligo-attached magnetic beads. Fragmentation was performed using divalent cations under elevated temperature in Illumina proprietary fragmentation buffer. First strand cDNA was synthesized using random oligonucleotides and SuperScript II (Invitrogen). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities and enzymes were removed. After adenylation of 3’ ends of DNA fragments, Illumina indexed PE adaptor oligonucleotides were ligated. DNA fragments with ligated adaptor molecules were selectively enriched using Illumina PCR primer PE1.0 and PE2.0 in a 15 cycle PCR reaction. Size-selection and purification of cDNA fragments with preferentially 200–300 bp in length was performed using SPRIBeads (Beckman Coulter). The size distribution of cDNA libraries was measured using the high sensitivity D1000 assay on the 2200 TapeStation instrument (Agilent). cDNA libraries were quantified using KAPA Library Quantification Kits (Kapa Biosystems). After cluster generation on a cBot, a 75-bp single-end run was performed on Illumina HiSeq1500.

After base calling and de-multiplexing using CASAVA version 1.8, the 75-bp single-end reads were aligned to the human reference genome hg19 from UCSC by HISAT version 0.1.7–β using the default parameters. After mapping of the reads to the genome, we imported the data into Partek Genomics Suite v6.6 (PGS) to quantify the number of reads mapped to each gene annotated in the RefSeq hg19 annotation downloaded in November 2015. These raw read counts were used as input to DESeq2 for calculation of normalized signal for each transcript using the default parameters. After DESeq2 normalization, the normalized read counts were imported back into PGS and floored by setting all read counts to at least a read count of 1. Subsequent to flooring, all transcripts having a maximum over all groups means lower than 10 were removed. After dismissing the low expressed transcripts the data comprised of 13,108 transcripts. RNA-seq data can be accessed under GSE (GEO Submission, GSE92852; NCBI tracking system #18204865).

Statistical and descriptive bioinformatics of transcriptome data

An ANOVA model was performed to calculate the 1,000 most variable and the differentially expressed (DE) genes between all groups. DE genes were defined by an FC > 2 or < −2 and a FDR adjusted p-value < 0.05. To visualize the structure within the data, we performed PCA on all genes and HC of the 1,000 most variable genes with default settings in PGS based on the p-values according to the expression values of the samples across all conditions.

To define differences and similarities in transcript expression among the groups, we performed CENA based on Pearson’s correlation coefficients with a cutoff of r = 0.95 by using BioLayout Express3D. The union of DE genes (3,504 genes) from the two comparisons LPS-treated IL-10-APC versus LPS-treated moDC and IL-10-APC versus immature moDC was used as the input for the network analysis. We calculated group FC for each gene by comparing the mean expression of each group with the mean expression across all samples. Genes within the network, represented as nodes, were colored accordingly to determine a treatment-dependent structure in the expression data. To assess the functionality of specifically upregulated gene sets, we identified genes with a group FC > 1 and performed GOEA using the gene ontology consortium online platform (http://geneontology.org/page/go-enrichment-analysis).

Moreover, mean log2-ratios from the two comparisons LPS-treated IL-10-APC versus LPS-treated moDC and IL-10-APC versus immature moDC were visualized for the union of differentially expressed genes (3,504 genes) in a scatter plot. In addition, GOEA was performed on this gene set. Genes connected to the most significantly enriched GO term “Immune system process” were identified (black outline) and those within colored areas were again analyzed for GO term enrichment. Subsequently, genes corresponding to selected, significantly enriched GO terms were marked by color. Mean expression
values of selected key genes for all conditions were z-transformed and shown in a heatmap. Furthermore, these genes were grouped according to their functionality based on connected GO terms and literature references.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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Author contributions
AH and SH designed the research, performed experiments, analyzed the results, prepared figures, and wrote the manuscript. JFAW, SND and KR performed the experiments. KK contributed to data analysis, supervision and wrote the manuscript. TU contributed to data analysis and supervision. NS prepared the data and performed basic analysis. JLS wrote the manuscript. PB designed the research, analyzed the results and wrote the manuscript.

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