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Human monocytic myeloid-derived suppressor cells impair B-cell phenotype and function in vitro

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Myeloid-derived suppressor cells (MDSCs) are key regulators of immunity that initially have been defined by their ability to potently suppress T-cell responses. Recent studies collectively demonstrate that the suppressive activity of MDSCs is not limited to T cells, but rather affects a broad range of immune cell subsets. However, relatively few studies have assessed the impact of MDSCs on B cells, particularly in the human context. Here, we report that human monocytic MDSCs (M-MDSCs) significantly interfere with human B-cell proliferation and function in vitro. We further show that the inhibition occurs independent of direct cell-contact and involves the expression of suppressive mediators such as indoleamine 2, 3-dioxygenase (IDO), arginase-1 (Arg1), and nitric oxide (NO). In addition, our studies demonstrate that the suppression of B cells by M-MDSCs is paralleled by a skewing in B-cell phenotype and gene expression signatures. M-MDSCs induced the downregulation of key surface markers on activated B cells, including IgM, HLA-DR, CD80, CD86, TACI, and CD95. Concurrently, M-MDSCs but not conventional monocytes elicited alterations in the transcription of genes involved in apoptosis induction, class-switch regulation, and B-cell differentiation and function. In summary, this study expands our understanding of the regulatory role of M-MDSCs for human B-cell responses.

Keywords: Immune regulation · Immune suppression · B-cell responses · B-cell phenotype · Monocytic myeloid-derived suppressor cells

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that comprise different phenotypes and possess potent suppressive capacities. Under pathological conditions such as chronic inflammation, infection, or cancer, abnormal myelopoiesis is advantaged through persistent stimulation and eventually results in accumulation of immature myeloid cells. Through a complex network of signals in the peripheral microenvironment, these cells acquire potent immunosuppressive properties and therefore are termed MDSCs [1, 2].

MDSCs have been extensively studied in mice and humans as regulators of innate and adaptive immunity. They are major contributors to progression and persistence of tumors in cancer patients, by locally suppressing antitumor responses [3, 4]. On the other hand, it is evident that MDSC-suppressive activity is indispensable for the maintenance of immune homeostasis and prevention of autoimmune diseases [5, 6].

Phenotypically, two different subsets of MDSCs are defined according to their morphology and surface marker expression, namely polymorphonuclear (PMN-) and monocytic (M-) MDSCs, being $CD11b^+CD15^+CD33^+HLA\text{-}DR^-$ and $CD11b^+CD14^+CD33^+HLA\text{-}DR^-$ in humans, respectively [6, 7]. Functionally, MDSCs were initially identified by their characteristic ability to suppress T-cell proliferation and function. The molecular mechanisms of inhibition applied by MDSCs have been shown to involve both, signals mediated through direct cell-contact and secretion of soluble mediators. PMN-MDSC-suppressive mechanisms mainly involve the deprivation of L-arginine by expression of arginase-1 (Arg1) and the production of reactive oxygen species (ROS). By contrast, M-MDSCs primarily produce high levels of nitric oxide (NO) via inducible nitric oxide synthase (iNOS) and secrete inhibitory cytokines such as IL-10 [7–9]. Studies also demonstrate that MDSCs promote de novo generation of regulatory T cells (T_{regs}), especially in the tumor environment [10, 11]. Besides, MDSCs have been shown to inhibit T-cell responses through deprivation of L-tryptophan, mediated by the expression of indoleamine 2,3-dioxygenase (IDO) [12].

During the last few years it became clear that the suppressive impact of MDSCs does not remain restricted toward T-cell responses. MDSCs have been shown to interact with a wide range of immune cells including NK cells [13, 14], macrophages, neutrophils, and dendritic cells [15–17]. Despite the extensive expansion of research in this field, only few studies have discussed the interaction of MDSCs and B cells so far. Present studies on the role of MDSCs during B-cell responses mainly deal with murine models [18]. In 2015, Crook and colleagues showed that MDSCs efficiently inhibit B-cell proliferation and antibody production in a murine model of rheumatoid arthritis [19]. Examination of a retrovirus-induced murine AIDS model revealed NO- and ROS-dependent suppression of B-cell responses through MDSCs in a contact-independent manner [20]. Additionally, MDSCs directly impair B-cell differentiation during tumor progression in mice [21].

Several human B-cell driven diseases such as multiple myeloma (MM) and chronic lymphocytic leukemia (CLL) go along with a significant increase in MDSC-expansion [22, 23]. Moreover, enhanced numbers of circulating MDSCs have been shown to correlate with cancer progression and poor disease outcome in B-cell lymphoma patients [24, 25]. As these cells are able to modulate a wide variety of immune responses under differential conditions, a direct effect of MDSCs on B cells during human B-cell malignancies cannot be excluded, especially considering the murine studies on MDSC-B cell interactions listed above. Thus, there is a requirement for more experimental setups dealing with the impact of MDSCs on B-cell functionality specifically in the human system.

Recently, our group demonstrated for the first time that PMN-MDSCs isolated from human peripheral blood significantly interfere with B-cell proliferation, viability, and antibody production. We found that in vitro modulation of B-cell responses by PMN-MDSCs mainly involves Arg1 activity and production of NO and ROS [26]. According to our knowledge, there exist no studies dealing with the interaction of human M-MDSCs and B cells so far. However, it is mainly the $CD14^+HLA\text{-}DR^-$ monocytic subset of MDSCs that has been found to be expanded in patients suffering from proliferative B-cell diseases [22].

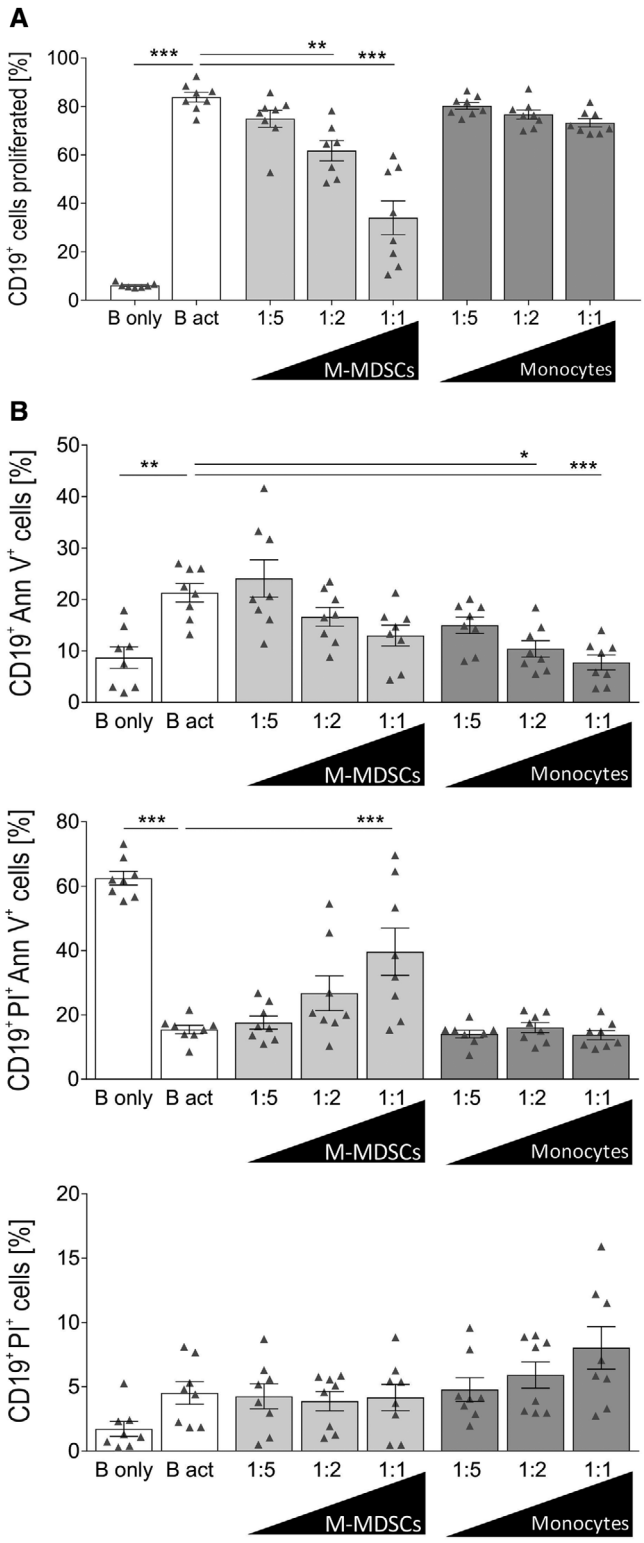
In the current study, we show that human M-MDSCs efficiently suppress proliferation and function of human B cells in a contact-independent manner by NO, Arg1, and IDO. We further demonstrate that M-MDSC-mediated suppression of B cells is accompanied by alterations within B-cell subsets and deregulation of B-cell specific gene expression.

Results

M-MDSCs efficiently suppress proliferation of B cells in a concentration-dependent manner

Besides the expression of a characteristic pattern of myeloid surface markers, MDSCs are functionally defined by their ability to suppress T-cell proliferation in vitro [1]. As mentioned above, there exist no studies concerning the proliferative behavior of human B cells in the presence of monocytic MDSCs. Thus, we initially examined whether $CD14^+HLA\text{-}DR^-$ MDSCs isolated from human peripheral blood were able to inhibit proliferation of activated B cells in vitro by co-culturing them in three different ratios (M-MDSC: B cells 1:5, 1:2, and 1:1). As a control, enriched B cells were additionally cultured with $CD14^+HLA\text{-}DR^+$ conventional monocytes to ensure that any observation relied on M-MDSC-specific mechanisms.

As depicted in Figure 1A, our first experiment revealed that M-MDSCs efficiently diminished the proliferation of B cells in a concentration-dependent manner, whereas conventional monocytes did not. M-MDSC-mediated suppression of proliferation was statistically significant when M-MDSCs and B cells were cultured at a ratio of either 1:2 or 1:1, with the most strongly marked effects observed at ratio 1:1 (Fig. 1A).



When assessing B-cell death via PI staining, we found that neither the presence of M-MDSCs nor conventional monocytes significantly altered the frequency of PI^+ cells within the overall $CD19^+$ population. Yet, co-culture of M-MDSCs led to an increase in the overall percentage of Annexin V and PI double-positive, late apoptotic B cells in a concentration-dependent manner (Fig. 1B).

M-MDSC reduce the IgM-Production of activated B cells in vitro

The main effector function of activated B cells is the production and secretion of protective antibodies, targeting pathogen or danger-associated epitopes. Antibody responses as a first-line defense can take place rapidly within a few hours in a T-cell independent (TI) manner. B-cell activation through TI antigens (e.g. CpG) mainly induces the production of IgM [27].

Hence, we set out to investigate IgM levels in the supernatant of activated B cells, either in the absence or presence of M-MDSCs/monocytes. Figure 2 illustrates that in vitro IgM-secretion of B cells was enhanced upon TI activation. M-MDSCs decreased B cell-mediated IgM-secretion in a concentration-dependent manner, although statistically not significant as defined by ANOVA multiple comparisons. However, when performing Student's *t*-test for comparison of groups, inhibition of IgM-production by M-MDSCs was statistically significant at culture ratio 1:1 (p-value = 0.0046). This finding did not apply to the co-culture of monocytes (Fig. 2).

M-MDSC-mediated suppression of B-cell proliferation occurs contact independently

In the course of immune suppression, MDSCs mediate signals via direct cell-contact or by secretion of inhibitory molecules. We recently found that efficient suppression of B cells by PMN-MDSCs required direct cell-contact [26]. To determine whether

Figure 1. M-MDSCs significantly suppress proliferation of B cells in a concentration-dependent manner. An enriched population of resting B cells and $CD14^+HLA\text{-}DR^-$ M-MDSCs or $CD14^+HLA\text{-}DR^+$ monocytes were isolated from human peripheral blood and co-cultured in a ratio of 1:5, 1:2, and 1:1 (M-MDSC/MONOS: B cells). B-cell stimulation was performed in a TI manner for 120 h using 1 μg/mL CpG-ODN DNA and 5 μg/mL $F(ab')_2$ IgM fragment. As a control, activated B cells (B act) and unstimulated B cells (B only) were cultured separately. (A) B cells were stained with CFSE prior to culturing and with PE-linked anti-CD19 antibody after 5 days of stimulation. Proliferation rate of $CD19^+$ cells was assessed by analysis of CFSE-intensity. (B) Cells were harvested and stained with PE-linked anti-CD19 antibody, APC-linked Annexin V and PI after 5 days of stimulation. B-cell death and apoptosis were determined using flow cytometry. The upper panel shows Annexin V^+ (early apoptotic) cells, the middle panel depicts Annexin V^+ PI^+ (late apoptotic) cells and in the lower panel, PI^+ (death) cells are shown. For the gating strategy applied, please refer to Supporting Information Figure 1A. Bar graphs show $n = 8$ independent experiments. Significance was determined by one-way analysis of variance (ANOVA) followed by ANOVA multiple comparisons with Bonferroni correction ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Error bars represent the mean ± SEM.

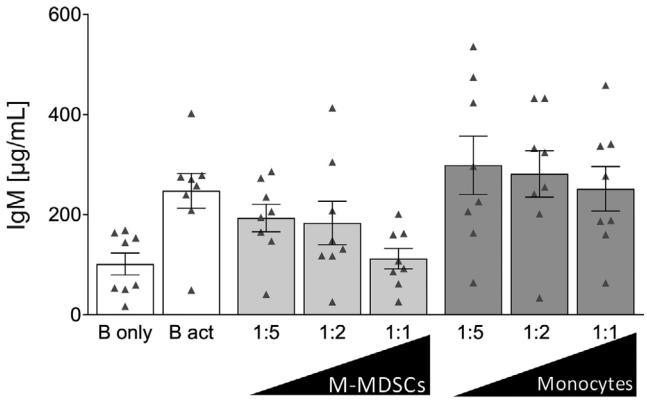


Figure 2. M-MDSCs significantly reduce the IgM-production of activated B cells in vitro. Cells were cultured and stimulated as described in Figure 1. Supernatants were collected 120 h post-stimulation and freezed at –20°C. Prior to ELISA performance, supernatants were diluted 1:100 in ELISA-assay buffer. ELISA was conducted using the human IgM Ready-SET-GO Kit by eBioscience according to manufacturer's protocol. Bar graphs depict the concentration of IgM in µg/mL, reflecting $n = 8$ independent experiments. Significance was determined by one-way analysis of variance (ANOVA) followed by ANOVA multiple comparisons with Bonferroni correction ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Error bars represent the mean ± SEM.

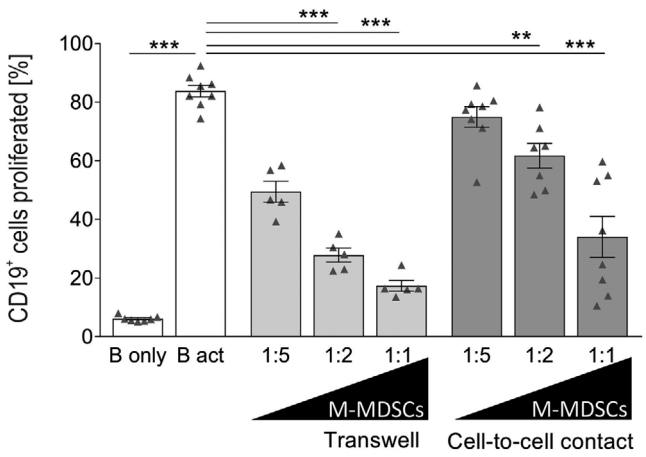


Figure 3. M-MDSC-mediated suppression of B cells occurs contact independently. An enriched population of resting B cells and $CD14^{+}$ $HLA\text{-}DR^{-}$ M-MDSCs isolated from human peripheral blood were co-cultured in a ratio of 1:5, 1:2, and 1:1 (M-MDSC/MONOS: B cells). Direct physical cell-contact of B cells and M-MDSCs was prevented by using transwell-system inserts (B cells cultured only in the bottom wells, M-MDSCs cultured in the upper well-inserts). B-cell stimulation was performed in a TI manner for 120 h using 1 µg/mL CpG-ODN DNA and 5 µg/mL $F(ab')_2$ IgM fragment. As a control, activated B cells (B act) and unstimulated B cells (B only) were cultured separately. B cells were stained with CFSE prior to culturing and with PE-linked anti-CD19 antibody after 5 days of stimulation. Proliferation rate of $CD19^{+}$ cells was assessed by analysis of CFSE-intensity. Bar graphs depict $n = 5$–8 independent experiments. Significance was determined by one-way analysis of variance (ANOVA) followed by ANOVA multiple comparisons with Bonferroni correction ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Error bars represent the mean ± SEM.

M-MDSC-mediated inhibition of B cells was also dependent on cell-contact, we performed transwell-system assays. As a control, the suppression assay was simultaneously performed without transwell-inserts, allowing direct contact.

As illustrated in Figure 3, physical separation of B cells and M-MDSCs through transwell-inserts did not interfere with M-MDSC-suppressive capacity. In the absence of direct cell-contact, the M-MDSC-induced decrease of B-cell proliferation could still be observed in its full potency, as it could be seen under cell-contact conditions (Fig. 3).

M-MDSC-inhibitory mechanisms include enzymes involved in nitric oxide metabolism

As we could work out that M-MDSC-dependent inhibition of B cells did not rely on direct cell-contact, we assumed that soluble factors in the culture supernatant may mediate suppression. This would be in consistence with previous studies, reporting that M-MDSCs mainly produce NO, prostaglandin E_2, and inhibitory cytokines [7]. Thus, we applied several established inhibitors to the isolated M-MDSCs to figure out which molecular mechanisms were relevant to their suppressive capacity in our setting.

Upon addition of ROS-inhibitor diphenyleneiodonium chloride (DPI), there were no alterations in the M-MDSC-suppressive impact on B-cell proliferation. However, treatment with the inhibitors 1-methyl-tryptophan (1-MT), Nω-hydroxy-nor-arginine (Nor-NOHA), or L-NG-monomethyl arginine citrate (L-NMMA) interfering with IDO, Arg1, and iNOS, respectively, significantly restored B-cell proliferation (Fig. 4). Inhibition of Arg1 or iNOS completely restored B-cell proliferation, indicating that both enzymes were indispensable for M-MDSC-suppressive activity. IDO-inhibition could restore proliferation in a statistically significant manner but did not lead to an overall recovery of proliferation (Fig. 4).

In summary, these results suggest that M-MDSC-suppressive mechanisms in the course of B-cell inhibition may include the production of NO via iNOS as well as the deprivation of L-tryptophan and L-arginine from the culture, by expressing IDO and Arg1, respectively.

M-MDSCs lead to distinctive changes in B-cell surface markers

Consequently, we analyzed a wide range of markers involved in B-cell activation, to find out whether M-MDSCs modify the phenotype of $CD19^{+}$ cells in vitro. We could not detect any significant changes in the expression of B-cell activating factor receptor (BAFFR), CD69, IgD, or CCR7 (Supporting Information Fig. 2).

However, there was a significant decrease in the expression of several surface molecules on B cells upon co-culture of M-MDSCs, but not conventional monocytes. These include surface-IgM, human leukocyte antigen (HLA)-DR, CD80, CD86, transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI) and CD95 (Fig. 5A–F). Surface expression was

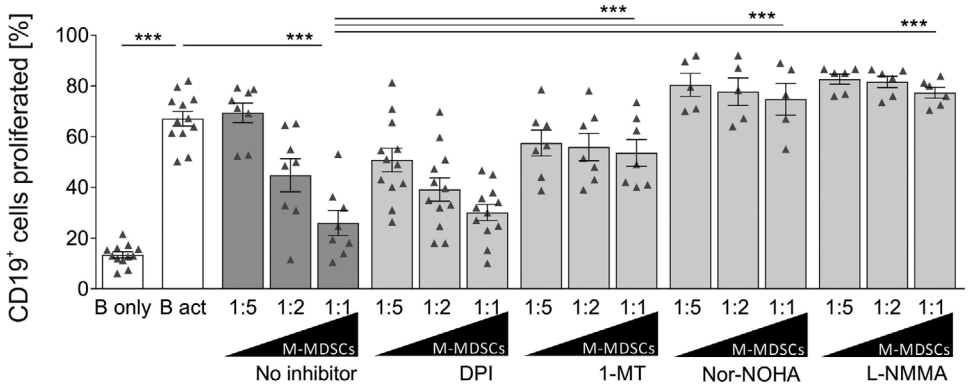


Figure 4. M-MDSC-inhibitory mechanisms involve indoleamine 2,3-dioxygenase (IDO), arginase-1 (Arg1), and inducible nitric oxide synthase (iNOS). Cells were cultured and stimulated as described in Figure 1. Prior to co-culturing, M-MDSCs were incubated with inhibitors in respective concentrations for 1 h (DPI: 10 μM, 1-MT: 1 mM, Nor-NOHA: 1 mM, and L-NMMA: 1 mM). B cells were stained with CFSE prior to culturing and with PE-linked anti-CD19 antibody after 5 days of stimulation. Proliferation rate of $CD19^+$ cells was assessed by analysis of CFSE-intensity. Bar graphs depict $n = 5–12$ independent experiments. Significance was determined by one-way analysis of variance (ANOVA) followed by ANOVA multiple comparisons with Bonferroni correction ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Error bars represent the mean ± SEM.

analyzed by determination of both, the median of fluorescent intensity (MFI) as well as the percentage of positive cells relative to the entire $CD19^+$ population (Fig. 5).

M-MDSCs induce phenotypic changes within specific B-cell subsets

As we have found that M-MDSCs induce significant changes in B-cell surface expression, we next investigated the influence of M-MDSCs on B-cell differentiation upon thymus-dependent (TD) stimulation, additionally including IL-21 and CD40L [28]. We examined selected B-cell populations in the culture relative to all $CD19^+$ cells. For each experimental trial, activated B cells were additionally cultured with conventional monocytes to ensure M-MDSC-specificity of observations.

Initially, we focussed on B-1 like cells that are known to respond fast as a first-line defense in mice models upon stimulation [29]. Figure 6 illustrates the percentage of human B-1 like cells, defined by surface expression of CD19, CD20,CD27, CD43, and lack of surface-CD70. Gating was based on the phenotype definition postulated by Rothstein et al., however, the existence and phenotype of human B-1 cells is still controversially discussed [30, 31]. According to the expression of CD5, we distinguished B-1a ($CD5^+$) from B-1b ($CD5^-$)-like cells.

The presence of M-MDSCs did not lead to significant changes in the percentage of B-1a-like cells if compared to activated B cells alone (Fig. 6A). Surprisingly, co-culturing of monocytes induced a concentration-dependent increase in B-1a like cell proportions (Fig. 6A). TD activation of $CD43^-$ B cells significantly induced a B-1b-like-phenotype in vitro and interestingly, the presence of M-MDSCs suppressed this induction in a concentration-dependent manner (Fig. 6B).

As murine B-1 cells are known to quickly differentiate into antibody-secreting cells (ASCs) upon their activation, we examined the percentage of plasmablasts (defined as IgD^- $CD27^{high}$ $CD38^{high}$ $CD138^-$) and plasma cells (defined as IgD^- $CD27^{high}$ $CD38^{high}$ $CD138^+$) [32]. We found that M-MDSC suppress both the emergence of plasmablasts (Fig. 6C) as well as plasma cells (Fig. 6D) when co-cultured in a ratio of 1:2 or 1:1. This was not true upon co-culture with monocytes, indicating that suppression of ASCs was a feature specific to M-MDSCs. The suppression of plasma cells occurred in a statistically significant manner.

Finally, we examined the B-cell differentiation into memory cells upon TD activation in vitro. Figure 6E demonstrates that $IgD^-CD27^+IgM^+$ memory cells were slightly induced upon activation of B cells. At the same time, we found that M-MDSCs considerably dampened the development of IgM^+ memory B cells, whereas conventional monocytes did not (Fig. 6E). The same was true for $IgD^-CD27^+IgM^-$ switched memory B cells (Fig. 6F). In both cases, alterations were not significant as determined by ANOVA.

Complementary, we investigated the susceptibility of B-cell subsets toward M-MDSC co-culture in terms of cell death by analyzing the percentage of dead (*zombie*$^+$) cells within each subpopulation. We found that M-MDSCs specifically induce cell death in antibody-secreting cells (both plasmablasts and plasma cells), whereas the rate of dead B-1 cells and memory B cells remained mostly unaltered (Supporting Information Fig. 3). These data demonstrate that there is a differential susceptibility of B cells toward M-MDSC-mediated inhibition, depending on the specific type of B-cell subset.

M-MDSCs induce changes in B-cell specific gene signatures

As our data demonstrated extensive influence of M-MDSCs on B cells on the cellular level, we performed genetic analysis of B cells for determination of their genetic profile. The gene array

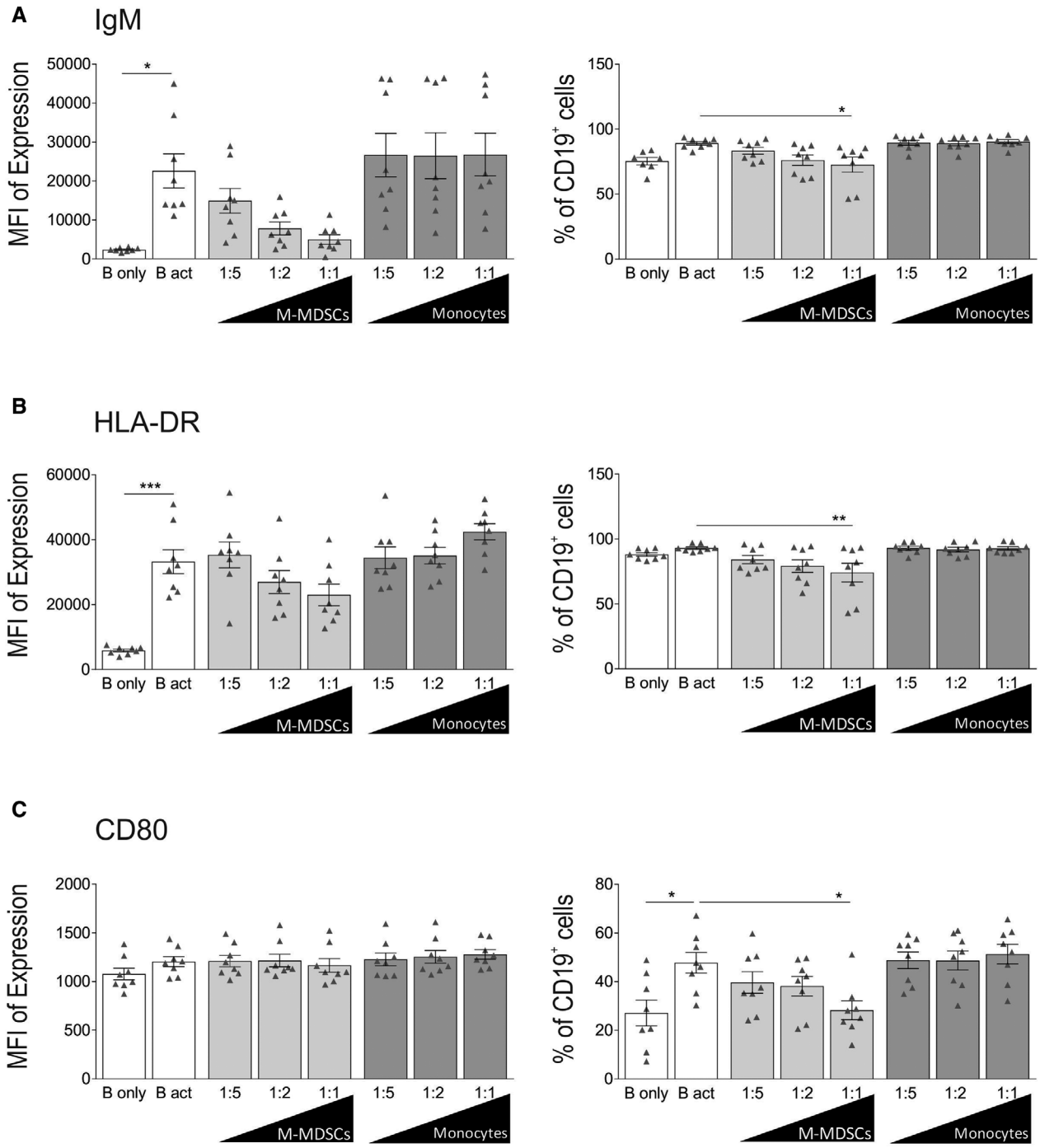


Figure 5. M-MDSCs lead to distinctive changes in B-cell surface expression. Cells were cultured and stimulated as described in Figure 1. Five days post-stimulation, cells were harvested and stained with antibodies targeting surface-CD19 and either (A) IgM, (B) HLA-DR, (C) CD80, (D) CD86, (E) TACI, or (F) CD95/FAS. The expression of respective surface molecules was analyzed using flow cytometry. Results are expressed as median fluorescence intensity (MFI; left graphs) and percentage of positive cells relative to the entire $CD19^+$ cell population (right graphs) for all markers. For the gating strategy applied, please refer to Supporting Information Figure 1B. Bar graphs show $n = 8$ independent experiments. Significance was determined by one-way analysis of variance (ANOVA) followed by ANOVA multiple comparisons with Bonferroni correction ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Error bars represent the mean ± SEM.

applied included 43 genes essentially involved in apoptosis and regulation of B-cell function. An expression profile was assessed for activated B cells alone or co-cultured with M-MDSCs or conventional monocytes. Figure 7 shows fold regulations in B cell-specific gene expression upon co-culturing with M-MDSCs (A) or monocytes (B), relative to activated B cells alone. Figure 7C summarizes up- and downregulation of genes within the three groups in a color-coded clustergram.

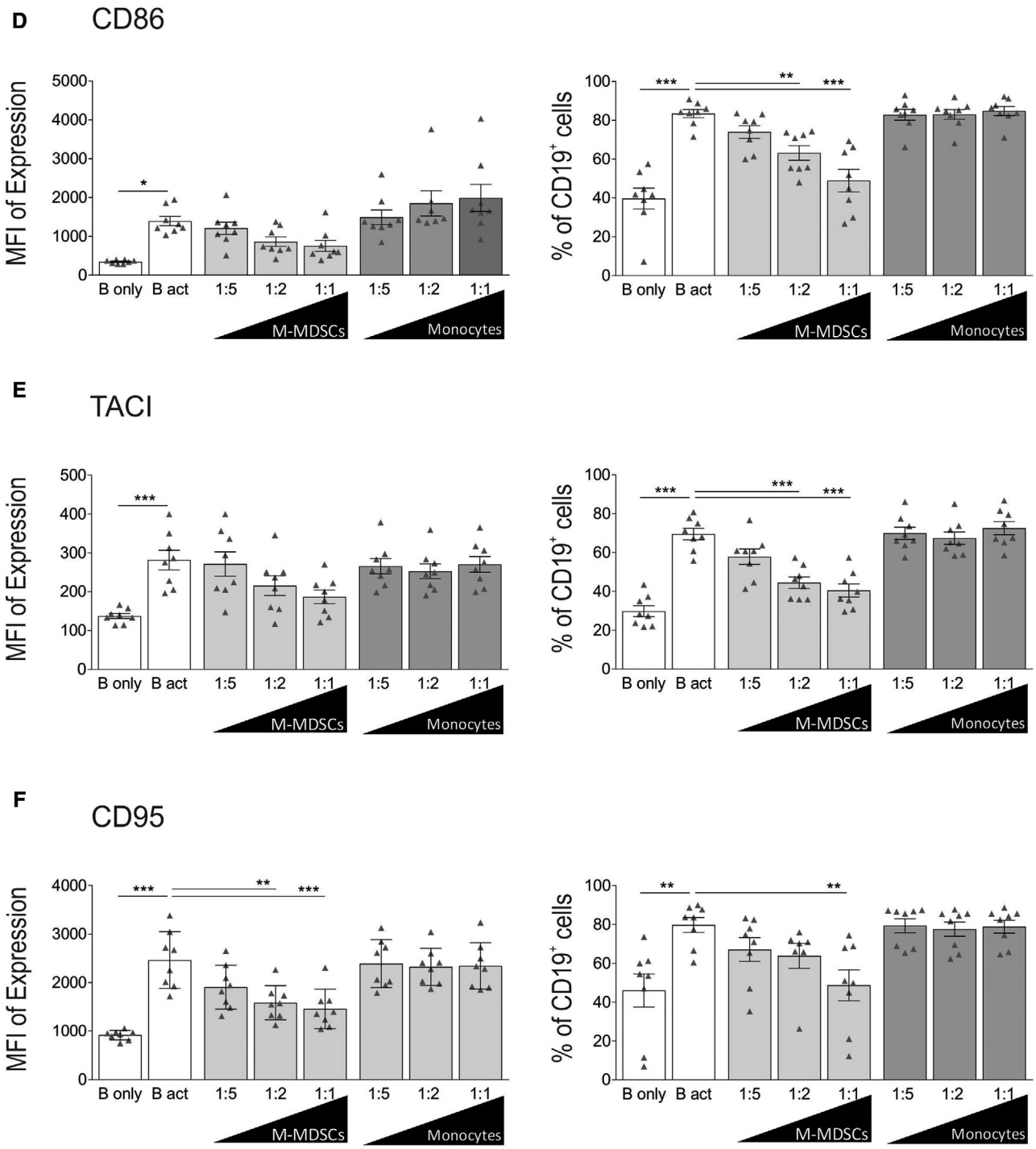


Figure 5. *Continued.*

We found one gene to be significantly downregulated in both B cells cultured with M-MDSCs as well as monocytes, namely induced myeloid leukemia cell differentiation protein 1 (Mcl-1). The simultaneous downregulation of Mcl-1 in both M-MDSCs and monocyte co-culture indicates an underlying mechanism specific for a broader range of monocytic or myeloid cells. As this observation was not specific to M-MDSCs, its interpretation is beyond the scope of this study.

Of interest, we found a total of five genes significantly upregulated in B cells after co-culture with M-MDSCs (*Bcl-2 associated X protein* (BAX), *B-cell CLL/lymphoma 2* (BCL-2), CD40, *FS-7-associated surface antigen* (FAS), and IL-6), but not conventional monocytes. Moreover, there were two genes significantly

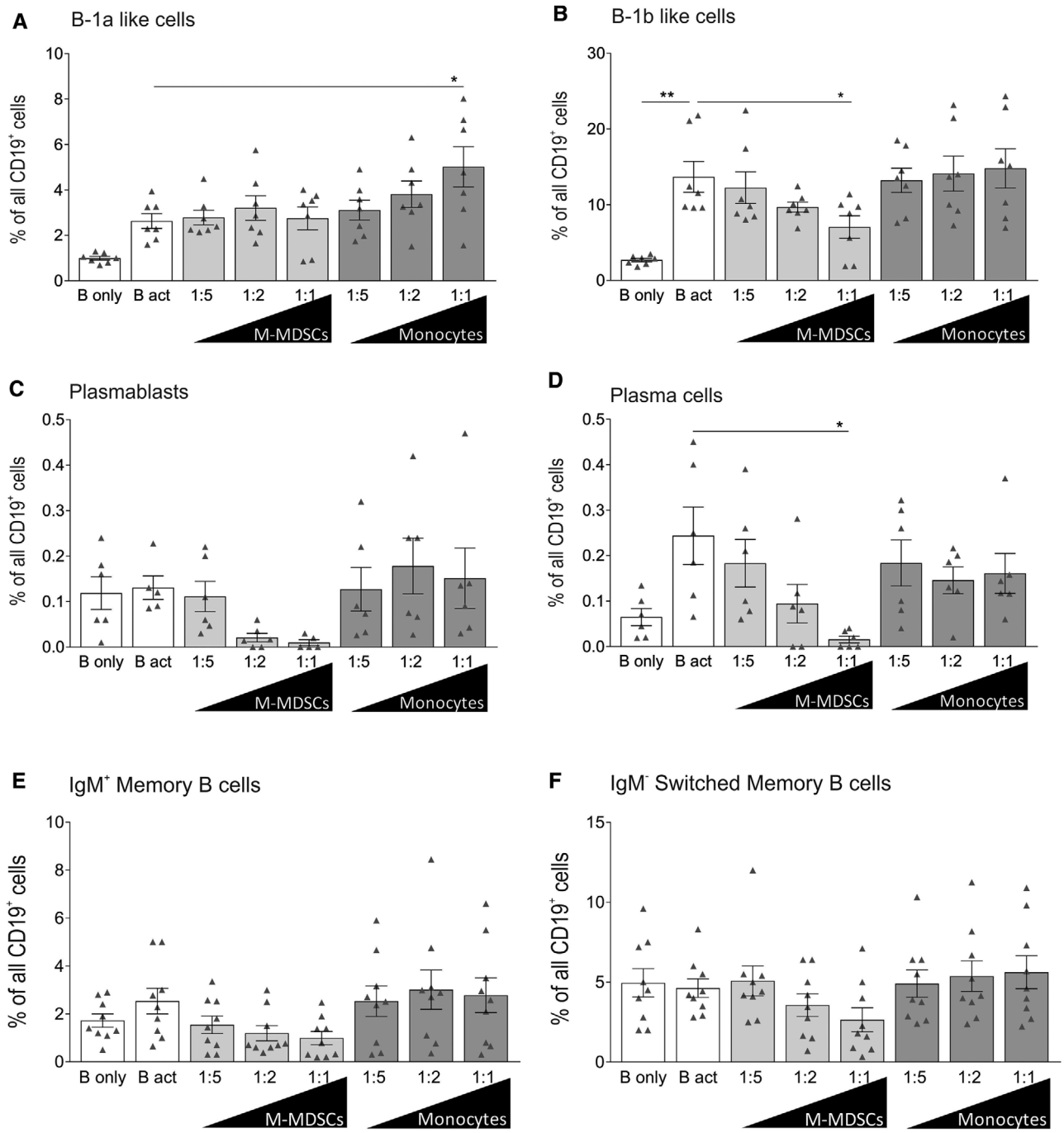


Figure 6. M-MDSCs induce phenotypic changes within specific B-cell subsets. Cells were cultured as described in Figure 1 and stimulated in a TD manner by adding 1 μg/mL CpG-ODN DNA, 5 μg/mL F(ab')$_2$ IgM fragment, 50 ng/mL IL-21, and 2 μg/mL CD40L for 120 h. Post-stimulation, cells were harvested and stained with antibodies targeting surface-CD19, CD20, IgM, IgD, CD43, CD27, CD38, CD138, CD70, CD5, and zombie-live/dead-dye. All phenotypes were adopted from Kaminski et al. B-1 like cells were defined as $CD19^+CD20^+CD43^+CD27^+IgM^+$ and distinguished into (A) B-1a ($CD5^+$) and (B) B-1b ($CD5^-$) cells. (C) Plasmablasts were defined as IgD^- $CD27^{high}$ $CD38^{high}$ $CD138^-$. (D) Plasma cells were defined as IgD^- $CD27^{high}$ $CD38^{high}$ $CD138^+$. (E) Non-switched memory B cells were defined as IgM^+ IgD^- $CD38^+$ $CD27^+$. (F) Switched memory B cells were defined as IgM^- IgD^- $CD38^+$ $CD27^+$. Percentage of B-cell subsets relative to all $CD19^+$ cells was determined using flow cytometry. For the gating strategy applied, please refer to Supporting Information Figure 1C. Bar graphs depict $n = 6$–9 independent experiments. Significance was determined by one-way analysis of variance (ANOVA) followed by ANOVA multiple comparisons with Bonferroni correction ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Error bars represent the mean ± SEM.

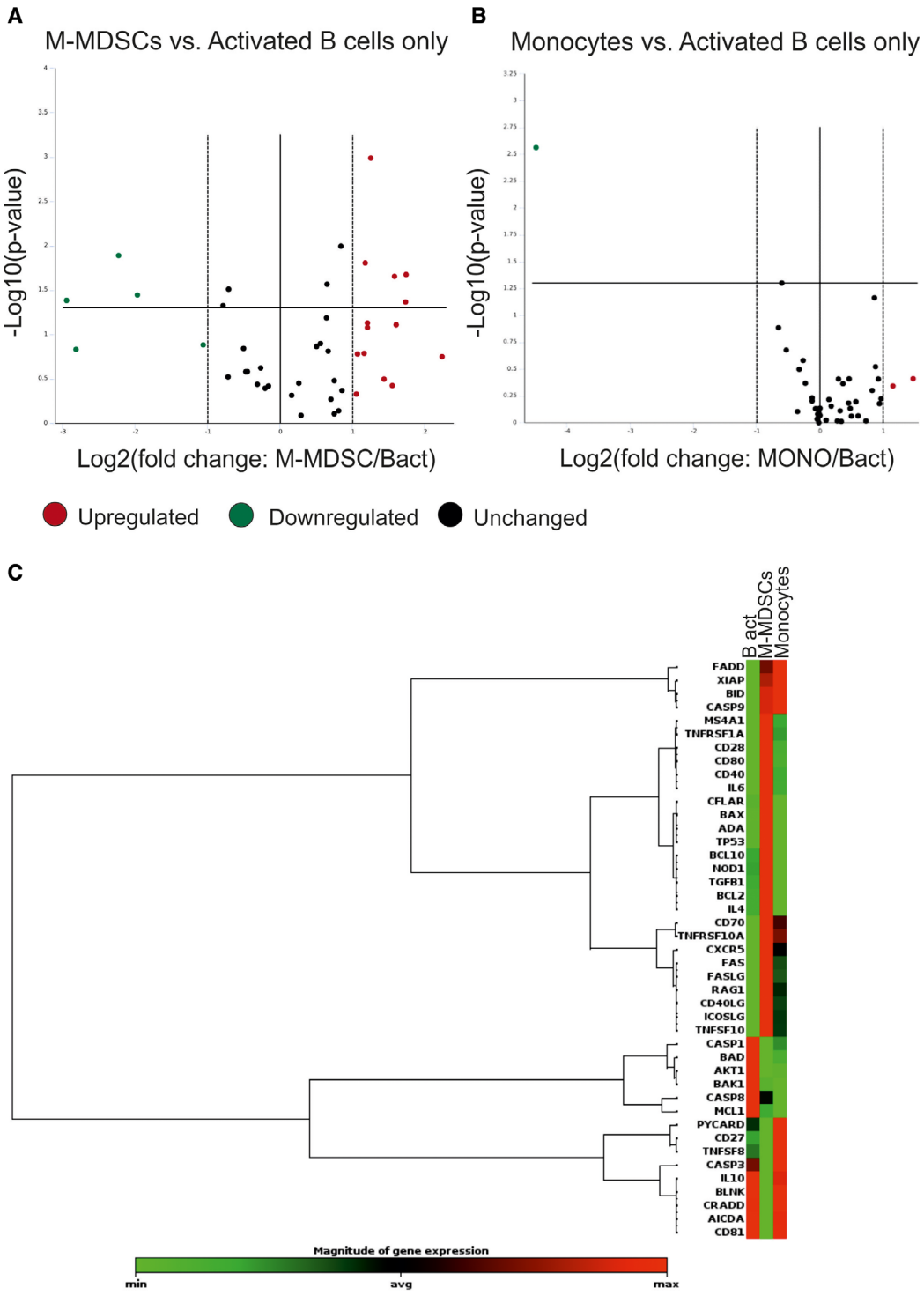


Figure 7. The presence of M-MDSCs leads to significant changes in the gene expression profile of B cells. An enriched population of resting B cells and $CD14^+$ $HLA\text{-}DR^-$ M-MDSCs isolated from human peripheral blood were co-cultured in a ratio of 1:1 (M-MDSC: B cells). Direct physical cell contact of B cells and M-MDSCs was prevented by using transwell-system inserts (B cells cultured only in the bottom wells, M-MDSCs cultured in the upper well-inserts). B-cell stimulation was performed in a TI manner for 120 h using 1 μg/mL CpG-ODN DNA and 5 μg/mL $F(ab')_2$ IgM fragment. As a control, activated B cells (B act) were cultured separately. Five days post-stimulation, pure B cells were harvested from the bottom wells and total RNA contents were isolated. Subsequently, RNA preamplification and transcription into cDNA was performed. RT-PCR was done using customer-specific RT^2 Profiler PCR Gene Expression Arrays by Qiagen (including 43 genes involved in apoptosis and B-cell function). Normalization and analysis of CP-values was done by array-specific analysis using the Data Analysis Centre provided by Qiagen.com. Results represent $n = 5$ independent experiments per group. (A and B) Fold-change in expression of respective genes is shown in a scatter plot for B cells co-cultured with M-MDSCs (A) or monocytes (B) as compared to activated B cells alone. (C) Up- or downregulation of gene expression within the three groups is depicted as color-coded clustergram.

Table 1. Fold regulation of genes that were found to be significantly deregulated in B cells upon co-culture with M-MDSCs as compared to B cells alone. Customer- and array-specific analysis of CP-values was performed using the Data Analysis Centre provided by Qiagen.com. Data represent $n = 5$ independent experiments. A p-value of <0.05 was considered statistically significant

Gene Abbreviation	Description	Fold-regulation in the presence of M-MDSCs
AICDA / AID	Activation-induced cytidine deaminase	−3.91
BAX	Bcl-2 associated X protein	+2.38
BCL-2	B-cell CLL/lymphoma 2	+2.26
CD40	TNF receptor superfamily, member 5	+3.34
FAS	TNF receptor superfamily, member 6	+3.33
IL-6	Interleukin-6	+2.99
IL-10	Interleukin-10	−7.69

underexpressed only in the co-culture containing M-MDSCs (*activation-induced cytidine deaminase* (AICDA) and IL-10). Intriguingly, the seven genes specifically deregulated in the presence of M-MDSCs are all essentially involved in the induction of apoptosis (such as BAX, BCL-2, and FAS), the regulation of class-switch and Ig-production (such as AICDA and CD40) or the differentiation and effector function of B cells (such as IL-6 and IL-10). Full gene descriptions and fold regulations in expression of respective genes are listed in Table 1 below.

Discussion

M-MDSC inhibit B cells in a contact-independent manner via IDO, Arg1, and iNOS

The relevance of the monocytic MDSC-subset with respect to regulation of B-cell responses has already been pointed out by many studies in mice [19, 20, 33]. Nevertheless, the direct effects of M-MDSCs on the proliferation and function of human B cells were elusive. Here, we report that M-MDSC-mediated modulation of B-cell immunity also applies in the human context. We found that M-MDSCs isolated from human peripheral blood efficiently suppress B-cell proliferation in a concentration-dependent manner (Fig. 1A). Alongside with the suppression of proliferation, the co-culture of M-MDSCs also decreased in vitro IgM-secretion of B cells upon stimulation with anti-IgM and CpG (Fig. 2). Importantly, as seen in Fig. 2, only M-MDSCs but not conventional monocytes interfered with IgM-production, proving that this effect was specific for HLA-DR$^-$ monocytic cells. These results conform to what we have reported previously in the context of PMN-MDSCs, indicating that both subsets of MDSCs are negatively regulating B-cell proliferation and IgM-production in humans [26].

We further show that inhibition of B-cell proliferation through M-MDSCs was not dependent on direct cell-to-cell contact, as inhibition still occurred upon physical separation of cells (Fig. 3). This finding is in agreement with murine studies, reporting that M-MDSCs predominantly convey suppression to B cells via secretion of soluble factors [34, 35]. At the same time, we identified IDO, Arg1, and iNOS as molecules involved in the contact-independent suppression by M-MDSCs, as supplementation of specific inhibitors significantly restored proliferation (Fig. 4). Of note, B-cell proliferation could be completely restored through inhibition of Arg1 and iNOS, whereas it was only partly restored upon IDO-inhibition. This suggests that Arg1 and iNOS are indispensable for M-MDSC-suppressive activity in this setting, while IDO may only partially mediate suppression.

Arg1 and iNOS are enzymes modulating the cellular metabolism through conversion of the amino acid L-arginine into urea and L-ornithine, or NO and L-citrulline, respectively [36]. Local deprivation of L-arginine is known to be a major mechanism applied by MDSCs to suppress T cells [1]. The depletion of L-arginine correlates with decreased global protein synthesis and eventually results in T-cell cycle arrest by modulation of cyclin-dependent kinases [37]. Moreover, the consumption of L-arginine through MDSCs has been reported to significantly interfere with T-cell receptor signaling [38]. On the other hand, NO produced by iNOS is an important factor implicated in MDSC-activity, as it negatively regulates intracellular proteins in target cells and induces apoptotic events [16, 36]. Our results point out that MDSC-derived NO potentially causes damage in B cells and thereby inhibits their function. This would be consistent with existing studies, describing NO-production as an inhibitory mechanism essentially involved in B-cell suppression by M-MDSCs in mice [19, 20]. The fact that inhibition of both, Arg1 or iNOS, completely abolished M-MDSC-mediated suppression indicates that a combination of both enzymes may come into play. Interestingly, the co-expression of Arg1 and iNOS is proposed to be a characteristic feature of suppressive myeloid cells [36]. For instance, cooperation of Arg1 and iNOS creates reactive nitrogen-oxide species such as peroxynitrite (PNT). PNT influences immune cell fate through biological modification of various signaling proteins such as Bcl-2 or FAS-ligand [36, 39].

Finally, the recovery of B-cell proliferation upon IDO-inhibition speaks for local L-tryptophan deprivation as an additional inhibitory mechanism. Concurrently, a previous study unveiled that MDSCs efficiently inhibit T cells through IDO-expression during breast cancer [40]. Here, we demonstrate that M-MDSC-mediated suppression of B cells equally involves the expression of IDO.

Thus, it is reasonable to hypothesize that not only T cells but also B cells are highly sensitive toward local nutrient depletion evoked by M-MDSCs. In addition, the production of NO and reactive nitrogen-oxide species probably interferes with B-cell functions in vitro. In summary, these results match to what we have previously found in our study dealing with PMN-MDSCs and together point to a role of both MDSC-subsets as negative regulators during human B-cell responses.

M-MDSCs induce distinct alterations in B-cell phenotypes

To intensify our understanding of M-MDSC suppressive mechanisms, we examined surface expression and phenotype of in vitro activated B cells upon co-culture. We detected downregulation of several surface markers in the presence of M-MDSC but not regular monocytes, namely IgM, HLA-DR, CD80, CD86, TACI, and CD95 (Fig. 5A–F). Alterations in surface expression were mainly manifested as reduced percentage of positive cells within the overall $CD19^+$ population. Generally, the downregulation of surface-IgM corresponds with dampened B-cell proliferation and IgM-secretion that is seen upon co-culture of M-MDSC. The significant reduction of $HLA\text{-}DR^+$, $CD80^+$, and $CD86^+$ B-cell subsets is of high interest, as these molecules are essentially involved in B cell-driven antigen-presentation (Fig. 5B–D) [41]. These results allow us to speculate that M-MDSCs may be able to interfere with the B-cell inherent capacity to present antigen to their cognate T cell.

As depicted in Figure 5E, TI stimulation of B cells leads to a marked upregulation of TACI-expression in vitro. However, upon addition of M-MDSCs, the percentage of $TACI^+$ B cells was significantly reduced (Fig. 5E). This finding is of high relevance, as the deregulation of TACI-expression has been associated with impaired B-cell function in several immune deficient patients [42, 43]. Moreover, TACI-deficiency in mice is accompanied by autoimmune events and severe defects in TI directed immune responses [44]. Hence, the MDSC-dependent modulation of TACI-expression in human B cells should be subject to prospective studies.

At last, M-MDSCs induced a significant reduction in the expression of the FAS death receptor (CD95), which is upregulated on B cells upon activation (Fig. 5F). Usually, FAS-induced apoptosis is initiated externally upon binding of soluble or membrane-bound FAS-ligand and can involve the internalization of the whole FAS-complex [45]. Thus, we speculate that human M-MDSCs may express FAS-ligand, binding to the FAS-receptor on B cells, thereby inducing its internalization and promote apoptosis. This would explain the downregulation of surface-CD95 as well as the enhanced rate of late apoptotic B cells in the M-MDSC co-culture. Alternatively, MDSC-derived factors such as NO and PNT have already been reported to directly modulate FAS-expression [36].

Our study additionally focused on the formation of three important B-cell subsets, namely innate B-1 like cells, ASCs, and memory B cells (Fig. 6). As shown in Figure 6A, the co-culture of monocytes promoted genesis of $CD5^+$ B-1a like cells, whereas M-MDSCs did not. On the other hand, M-MDSCs but not monocytes significantly decreased the number of $CD5^-$ B-1b like cells in a concentration-dependent manner (Fig. 6B). In conclusion, we show that human M-MDSCs and regular monocytes conditionally alter the phenotype of B-1 like cells, with monocytes probably having a promoting, M-MDSCs a suppressive effect. This is of high interest to further studies, as murine B-1 cells have been reported to be major players in maintenance of homeostasis and first line immune responses [29, 31].

When examining ASCs upon TD stimulation in vitro, we found similar rates of cells in the monocyte co-culture as compared to the positive control. By contrast, M-MDSCs strongly decreased the rate of plasmablasts (Fig. 6C) and significantly suppressed the emergence of plasma cells (Fig. 6D). The decrease in proportion of antibody-secreting cells was accompanied by a significant induction of B-cell death specifically within these two populations (Supporting Information Fig. 2). Notably, a recent study showed that PMN-MDSCs inhibit proliferation and function of $CD138^+$ B cells in the central nervous system, which is in accordance with our results [46]. As a consequence, we hypothesize that not only PMN-MDSCs but also M-MDSCs control B-cell effector function by suppressing plasma cell differentiation in humans.

Concerning memory B cells, we found slight reductions upon M-MDSC co-culture, but no significant changes, indicating that memory B cells were only mildly affected by M-MDSCs under these conditions (Fig. 6E and F).

M-MDSCs impact B-cell specific gene expression profiles

To uncover B cell genetic modifications upon M-MDSC co-culture, we evaluated the expression of 43 genes essentially involved in B-cell function and survival. Of note, B cells were physically separated from M-MDSCs via transwell-inserts prior to genetic analysis, meaning that following observations exclusively rely on contact-independent mechanisms. The gene array revealed a total of seven genes significantly deregulated in the presence of M-MDSCs but not conventional monocytes, again highlighting the individual function of M-MDSCs (Fig. 7).

We found three apoptotic regulators significantly upregulated in B cells cultured with M-MDSCs, namely BAX, Bcl-2, and FAS. BAX and Bcl-2 belong into the Bcl-2 protein family, whose members are known to be crucially involved in apoptosis prevention or initiation. BAX is a pro-apoptotic death effector triggering intracellular proteolysis by permeabilization of the outer mitochondrial membrane. In contrast, Bcl-2 is a pro-survival effector [47]. According to literature, the tightly regulated balance between pro- and anti-apoptotic Bcl-2 proteins in the cell decides whether apoptosis is initiated or not [48]. Hence, we suggest that the upregulation of Bcl-2 might be a compensatory mechanism, counteracting the induction of the death factor BAX in B cells upon M-MDSCs co-culture. The third apoptosis factor that we found deregulated on the transcriptional level was the death receptor FAS (CD95). As already discussed above, FAS-density on the B-cell surface was diminished by M-MDSCs in a concentration-dependent manner, which might be caused by receptor-internalization (Fig. 5F). Here, we found that at the same time, FAS-expression was significantly enhanced on the transcriptional level. Remarkably, a former study demonstrated that FAS can be directly triggered by NO in human cancer cells [49]. More precisely, NO was shown to disrupt a FAS-specific gene repressor in the promoter region, thereby inducing FAS-transcription [49]. Therefore, it is likely that M-MDSC-derived factors such as NO led to the boost of FAS-expression

in our experiment. Another possible explanation would be the production of FAS-protein as a compensatory response upon FAS-receptor internalization and degradation.

In addition, there were significant alterations in CD40 and AICDA expression of B cells upon co-culturing with M-MDSCs (Table 1). CD40 (TNFR super family member 5) is crucially involved in B-cell activation and communication with T cells in germinal centers. Upon binding of CD40-ligand, B cells acquire potent antigen-presenting capacities and stimulatory properties [41]. On the other hand, AICDA is an enzyme crucially involved in antigen diversification and class-switch recombination of Igs, as it induces gene conversion and somatic hypermutation [50]. Recent studies demonstrate that AICDA also plays an important role in developing B cells and TLR-mediated responses [51]. Here, we show that the presence of M-MDSCs increases CD40 and diminishes AICDA expression in TI stimulated B cells, implying that M-MDSCs may manipulate B cell-T cell interactions as well as Ig-production and class-switch.

Finally, the presence of M-MDSCs but not conventional monocytes induced changes in the B-cell intrinsic expression of the two key cytokines IL-6 and IL-10 (Table 1). Upon M-MDSC supplementation, transcription of IL-6 was significantly upregulated whereas IL-10 expression declined. Both IL-6 and IL-10 are highly pleiotropic signaling molecules implicated in the development and persistence of various immune diseases [52, 53].

Interestingly, MDSCs have been recently shown to prevent the accumulation of IL-6 and GM-CSF producing B cells during autoimmunity in the central nervous system [46]. However, when performing cytokine staining for $CD19^+IL\text{-}6^+$ B cells via flow cytometry, we could not detect any significant difference in intracellular IL-6 expression (Supporting Information Fig. 4). To draw any further conclusions about the role of IL-6 expression in this setting, future studies should additionally focus on IL-6 concentrations in the supernatant.

The downregulation of IL-10 transcription in B cells upon M-MDSC co-culture is of utmost interest, as $IL\text{-}10^+$ regulatory B cells have been shown to be important mediators of T-cell responses [54]. When analyzing the rate of $CD19^+IL\text{-}10^+$ cells using intracellular flow cytometry staining, we found slightly but not significantly reduced proportions of IL-10-expressing B cells in the co-culture of M-MDSCs (Supporting Information Fig. 5).

While the interplay of monocytic MDSCs and T cells is a well-investigated field, direct relations between M-MDSCs and B cells in the human system remained undescribed. Here, we demonstrate that isolated and activated human B cells are highly susceptible toward the co-culture of $HLA\text{-}DR^-$ M-MDSCs, but not $HLA\text{-}DR^+$ conventional monocytes. In this setting, M-MDSC-mediated suppression occurred contact-independently and involved the expression of IDO, Arg1, and iNOS. As a consequence, M-MDSCs significantly impaired B cells on various biological levels, including proliferation, IgM-production, phenotype, and gene expression signature. These findings are in correspondence to previous reports dealing with murine models and additionally indicate an essential role of M-MDSCs for human B-cell immunity. Since malignant B-cell transformations frequently go ahead with an increased number of circulating M-MDSCs in patients, their expansion has been suggested a prognostic factor of B-cell disease [25, 55]. Our results demonstrate a direct suppressive effect of $CD14^+HLA\text{-}DR^-$ MDSCs on human B cells, thereby identifying these cells as potential future therapeutic target for the treatment of B-cell malignancies.

Materials and methods

Cell isolation from human peripheral blood

The study was conducted at the University Children's Hospital and at the Institute for Pharmacology and Toxicology in Tübingen (Germany). All methods were approved by the local ethics committee. Buffy coats from healthy adult donors were provided by the DRK-Blutspendedienst Baden-Württemberg-Hessen Institute, Ulm, Germany. Therefore, gender and age of donors are not available.

PBMCs were prepared from blood samples by Ficoll density gradient sedimentation (Biochrome) and erythrocytes were lysed in premade lysis buffer (0.829% ammonium chloride, 0.1% potassium hydrogen carbonate, and 0.00372% EDTA disodium salt dihydrate in water). The lysed and washed PBMC-fraction was counted using the ABX MICROS CRP (ABX Diagnostics).

The B-cell isolation strategy was based on protocols previously established in our group, using negative selection from the PBMC-fraction [26]. Therefore, cells were labeled with FITC-conjugated anti-CD43 antibody followed by anti-FITC magnetic bead separation using the autoMACS® Pro Separator (Miltenyi Biotech). One further labeling step with a combination of anti-CD3 and anti-CD14 magnetic beads was performed, followed by a second magnetic separation step using the autoMACS® Pro Separator. The $CD43^-$ $CD3^-$ $CD14^-$ cell fraction represents an enriched population of resting B cells.

Isolation of M-MDSCs and monocytes was performed using both positive and negative selection from the PBMC-fraction. In brief, a positive selection for monocytic cells was done using anti-CD14 magnetic bead staining followed by magnetic bead separation using the autoMACS® Pro Separator. Subsequently, $CD14^+$ cells were labeled with anti-HLA-DR magnetic beads and were further separated into $HLA\text{-}DR^{+/-}$ cells in a second separation step with the autoMACS® Pro Separator. The $CD14^+$ $HLA\text{-}DR^+$ cell fraction represents conventional monocytes, whereas the $CD14^+$ $HLA\text{-}DR^-$ cell population constitutes M-MDSCs. All micro beads and antibodies involved in magnetic cell sorting were purchased from Miltenyi Biotech.

Cell culture, CFSE labeling, and B-cell activation

Isolated B cells were stained with carboxyfluorescein-succinimidyl ester (CFSE, Life Technologies) at day 0 according to the

manufacturer's protocol prior to culturing. Thymus-independent (TI) activation of B cells was performed by addition of 5 μg/mL anti-IgM (Jackson Immuno Research) and 2.5 μg/mL CpG ODN (InvivoGen). Thymus-dependent (TD) activation accessorily involved the addition of 50 ng/mL IL-21 and 2 μg/mL CD40L. A total of 5×10^5 B cells per well were seeded in a 48-well microtiter plate (Life Sciences) and either M-MDSCs or monocytes were co-cultured at the ratios 1:5, 1:2, or 1:1 (M-MDSC/monocytes: B cells). As a control, both activated B cells (B act) and unstimulated B cells (B only) were cultured separately. RPMI-1640 medium (Biochrome) was supplemented with 10% human serum, 2 mM L-glutamine (Millipore), and 100 U/mL penicillin/streptomycin (Millipore) for cell culture. After 120 h of stimulation at 37°C and 5% CO_2 in the Hera Cell Incubator (Heraeus), cells were harvested and supernatants were frozen at −20°C.

Transwell-assays were performed using transwell-system inserts (6.5 mm diameter inserts with 0.4 μm pores, Greiner BIO-ONE). B cells were cultured and activated in the bottom wells only, whereas M-MDSCs or monocytes were placed in the upper well-inserts.

Where indicated, inhibitors of arginase-1, iNOS, ROS, and IDO production, namely Nω-hydroxy-nor-arginine 1000 μM (Nor-NOHA/Enzo Life Sciences), L-NG-monomethyl arginine citrate 1000 μM (L-NMMA/Calbiochem), methyl-tryptophan 1000 μM (1-MT; Sigma–Aldrich) or diphenyleneiodonium chloride 10 μM (DPI; Sigma Aldrich) were incubated with M-MDSCs for 1 h at 37°C and 5% CO_2. Prior to co-culturing, M-MDSCs were washed to get rid of excessive inhibitors.

Flow cytometry

For B-cell staining, cells were harvested after 120 h of stimulation. To assess B-cell proliferation, the CFSE fluorescence intensity of $CD19^+$ cells was analyzed using PE-conjugated anti-CD19 antibody (Biolegend). For apoptosis and necrosis studies, PI (BD Bioscience) and Annexin V (BD Bioscience) stainings were performed according to manufacturer's protocol, respectively.

For analysis of B-cell surface marker expression, AlexaFluor700-conjugated anti-IgD, APC-conjugated anti-CD86 and anti-CD138, BV421-conjugated anti-CD19, FITC-conjugated anti-CCR7/CD197/CD80, PerCP-Cy5.5-conjugated anti-CD183, and anti-IgM and PE-Dazzle-conjugated anti-TACI/CD267 were purchased from Biolegend. APC-conjugated anti-CD95 and PE-conjugated anti-BAFFR/CD268 were obtained from eBioscience. FITC-conjugated CD69 and PerCP-conjugated HLA-DR were acquired from BD Bioscience. Prior to receptor staining, cells were incubated with UV anti-human Zombie dye (Biolegend) to allow exclusion of dead cells.

For phenotyping of B-cell subsets, AlexaFluor700-conjugated anti-IgD, APC-conjugated anti-CD138, and anti-IgM, BV421-conjugated anti-CD19, PE-Cy7-conjugated anti-CD27, PE-Dazzle-conjugated anti-CD20, PerCP-conjugated anti-CD38, and PerCP-Cy5.5-conjugated anti-CD70 were purchased from Biolegend. PE-conjugated anti-CD5 was obtained from Beckton Dickinson and FITC-conjugated anti-CD43 was bought from Miltenyi Biotech.

In case of intracellular staining protocols, cells were permeabilized prior to antibody-incubation using the Trancription Factor Buffer Set (BD Bioscience).

Flow cytometry measurements were performed with the BD FACS Calibur or the BD LSR II (BD Bioscience) and analysis was done using Flow Jo Version 10 (Three Star). For gating strategies applied to the data, please refer to Supporting Information Figure 1A–C as indicated in the figure captions. Throughout the procedures of sample preparation, measurement, and analysis, we have adhered to the "Guidelines for the use of flow cytometry and cell sorting in immunological studies" [56].

IgM ELISA

IgM analysis in collected supernatants was performed using human IgM Ready-SET-GO (eBioscience) according to the manufacturer's protocol. Supernatants were used in a 1:100 dilution. Absorbance reading was done with the ELx800 Absorbance Microplate Reader (BioTek).

Real-Time PCR

For gene expression studies, B cells and M-MDSCs/monocytes were co-cultured 1:1 without cell-contact using transwell-system inserts as described above. After 120 h of TI stimulation, transwell-inserts containing M-MDSCs/monocytes were removed, and pure B cells were harvested from the bottom wells. Cell lysis and isolation of total RNA-content was performed with the RNA Micro Kit (Qiagen). Subsequently, 50 ng of RNA per sample were utilized for array-specific pre-amplification followed by transcription into cDNA using the RT^2 PreAmp cDNA Synthesis Kit (Qiagen). Afterwards, RT^2 Profiler PCR Gene Expression Arrays were performed according to manufacturer's protocol using the RT^2 $SYBR^{TM}$ Green Master Mix (Qiagen). RT-PCR was run in the Light Cycler 480 (96-well block, Roche) and data were assessed under the '*second derivate max*' setting.

Customer-specific data analysis was performed with the Data Analysis Centre provided online by the Qiagen-homepage (https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/?akamai-feo = off).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism version 7.0. Differences between groups were determined by one-way analysis of variance (ANOVA) followed by ANOVA multiple comparisons with Bonferroni correction. p-Values < 0.05 were considered statistically significant (with $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). Error bars are depicted as mean ± SEM.

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Abbreviations: **AICDA**: activation-induced cytidine deaminase · **Arg1**: arginase-1 · **ASCs**: antibody-secreting cells · **BAX**: Bcl-2 associated X protein · **Bcl-2**: B-cell CLL/lymphoma 2 · **FAS**: FS-7-associated surface antigen · **HLA**: human leukocyte antigen · **iNOS**: Inducible nitric oxide synthase · **MDSCs**: Myeloid-derived suppressor cells · **M-MDSCs**: Monocytic myeloid-derived suppressor cells · **NO**: Nitric oxide · **PMN-MDSCs**: Polymorphnuclear myeloid-derived suppressor cells · **PNT**: Peroxynitrite · **ROS**: Reactive oxygen species · **TACI**: Transmembrane activator and calcium-modulating cyclophilin ligand interactor · **TI**: T-cell independent

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