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ORIGINAL ARTICLE

A Th1 cytokine–enriched microenvironment enhances tumor killing by activated T cells armed with bispecific antibodies and inhibits the development of myeloid-derived suppressor cells

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Abstract In this study, we investigated whether activated T cells (ATC) armed with bispecific antibodies (aATC) can inhibits tumor growth and MDSC development in a Th_1 cytokine–enriched (IL-2 and IFN-γ) microenvironment. Cytotoxicity mediated by aATC was significantly higher ($P < 0.001$) against breast cancer cell lines in the presence of Th_1 cytokines as compared with control co-cultures. In the presence of aATC, $CD33^+/CD11b^+/CD14^-/HLA\text{-}DR^-$ MDSC population was reduced significantly under both control ($P < 0.03$) and Th_1-enriched ($P < 0.036$) culture conditions. Cytokine analysis in the culture supernatants showed high levels of MDSC suppressive chemokines CXCL9 and CXCL10 in Th_1-enriched culture supernatants with highly significant increase ($P < 0.001$) in the presence of aATC. Interestingly, MDSC recovered from co-cultures without aATC showed potent ability to suppress activated T-cell-mediated cytotoxicity ($P < 0.001$), IFN-γ production ($P < 0.01$) and T-cell proliferation ($P < 0.05$) compared to those recovered from aATC-containing co-cultures. These data suggest that aATC can mediate enhanced killing of tumor cells and may suppress MDSC and T_{reg} differentiation, and presence of Th_1 cytokines potentiates aATC-induced suppression of MDSC, suggesting that Th_1-enriching immunotherapy may be beneficial in cancer treatment.

Keywords 3D culture model · Breast cancer · Activated T cells · Bispecific antibody · Peripheral blood mononuclear cells · Myeloid-derived suppressor cells

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Introduction

A growing body of evidence suggests that host immune cells with a suppressive phenotype limit the efficacy of immunotherapy regimens and facilitate tumor progression [1–4]. Among the suppressor cell types, regulatory T cells (T_{regs}), tumor-associated macrophages, and myeloid-derived suppressor cells (MDSC) are key suppressor cell populations that accumulate and mediate immune tolerance in tumors and secondary lymphoid tissues in hosts with advanced malignancies [5–7]. MDSC are defined as a $Lin^-HLA\text{-}DR^-CD33^+$ cells that are associated with disease progression and tumor burden and possess potent ability to suppress tumor-specific T-cell responses through the induction of T-cell anergy and the development of T_{regs} [8, 9]. Inhibition of MDSC function has been shown to delay tumor growth, suggesting that MDSC-mediated immune suppression can be reversed [10, 11].

Many cytokines, including interleukin 6 (IL-6), interleukin 13 (IL-13), granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte-stimulating factor (G-CSF) and interferon γ (IFN-γ), have been implicated in

the development and induction of effector functions of MDSC [9, 12–16]. Tumors themselves can release IL-6, IL-13, GM-CSF and G-CSF that in turn can lead to the recruitment and differentiation of granulocytic and monocytic precursors [17] possibly through the activation of the STAT3 signal transduction pathway [3, 18]. In our phase I clinical trial involving stage IV breast cancer patients who received activated T cells (ATC) armed with anti-CD3 × anti-Her2/*neu* bispecific antibody (Her2Bi), high levels of specific cytotoxicity by PBMC and circulating Th_1 cytokines were observed [19, 20]. Since Th_1 cytokine IFN-γ has been implicated in the induction and activation of MDSC, we asked whether: (1) a Th_1 cytokine–enriched (IL-2 and IFN-γ) microenvironment inhibits tumor growth and MDSC development; (2) Th_1-enriched microenvironment enhances targeted killing of tumor cells by Her2Bi-armed ATC (aATC); and (3) co-culture of tumor cells with aATC affects the development and suppressive ability of MDSC. Our data show that tumor spheres formed by breast cancer (BrCa) cell lines were visibly smaller in size in a Th_1-enriched microenvironment, and differentiation of granulocytic $CD14^-/HLA\text{-}DR^-/CD11b^+/CD33^+$ and monocytic $CD14^+/HLA\text{-}DR^-/CD11b^+/CD33^+$ MDSC populations was reduced with further reduction and attenuation of their suppressive activity in presence of aATC.

Materials and methods

Cell lines

The human breast cancer (BrCa) cell lines (SK-BR-3 and MDA-MB-231) were maintained in RPMI-1640 or DMEM culture media (Lonza Inc., Allendale, NJ) supplemented with 10% FBS (Lonza Inc.), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen).

Expansion and generation of ATC

$CD3^+$ T cells from PBMC were expanded using 20 ng/ml of OKT3 and 100 IU/ml of IL-2 for 14 days at a concentration of 1–2 × 10^6 PBMC/ml in RPMI-1640 supplemented with 10% FBS [21].

Production of anti-OKT3 × anti-Her2 bispecific antibodies

BiAb were produced by chemical heteroconjugation of OKT3 (a murine $IgG2_a$ anti-CD3 monoclonal antibody, Ortho Biotech, Horsham, PA) and Herceptin (a humanized anti-Her2 IgG_1, Genentech Inc., San Francisco, CA) as described [21]. Before use, ATC were armed with anti-CD3 × anti-Her2 (Her2Bi) bispecific antibodies (aATC) using a previously optimized concentration of BiAb (50 ng/10^6 ATC) for 30 min.

3D culture in matrigel

Cells were prepared at a concentration of 2,500 cells/ml in RPMI-1640 or DMEM culture media. Single cells are overlaid on a solidified layer of matrigel measuring approximately 1 mm in thickness as described [22]. Wells were coated with 100% matrigel in 0.25-ml aliquots in 24-well glass bottom plates and allowed to solidify by incubating at 37°C for 30 min. Breast cancer cells were then seeded onto the matrigel base as a single-cell suspension in the medium containing 2% matrigel, in the presence or absence of Th_1 cytokines (10 ng/ml IFN-γ and 100 IU/ml IL-2). After 5–7 days when tumor spheres were formed, PBMC were added at 10:1 ratio (10 PBMC/1 tumor cell). Her2Bi-armed ATC were added after 7 days of tumor cell and PBMC 3D co-culture at 10:1 (10 aATC/1 tumor cell) ratio (Fig. 1). The medium was replaced every 4 days. Tumor spheres were visualized in 5–7 days in 3D culture. In selected experiments, recombinant human MIG/CXCL9 (100 ng/ml) and IP-10/CXCL10 (100 ng/ml) were added to control cultures in the presence or absence of aATC.

Live cell imaging by inverted confocal microscopy using DiI and DiO dyes

Images were observed with a spinning disk microscope (Perkin Elmer UltraVIEW). Vybrant® DiI or DiO was added directly to normal culture media to uniformly label either cell suspensions (activated T cells) or adherent cells (BrCa) and incubated for 5 and 10 min at 37°C, respectively. Tumor spheres stained on the matrigel were washed thrice, similarly non-adherent ATC after loading were spun down, rinsed (3×) and resuspended in fresh medium before adding these cells to DiO-labeled tumor cells. Immunostained co-cultures were photographed using a Perkin Elmer UltraVIEW microscope. These dyes uniformly label cells via lateral diffusion in the plasma membrane and do not transfer from labeled to unlabeled cells. DiI (D3911) and DiO (D275) have fluorescence excitation and emission maxima separated by about 65 nm and thus can facilitate two-color labeling.

Cytotoxicity assay

Tumor cells were seeded in 24-well plate at 100,000 cells/well in volume of 1 ml. Cells were allowed to adhere followed by incubation with aATC for 3–5 days at 1:1 E/T in the presence or absence of Th_1 cytokines. At the end of

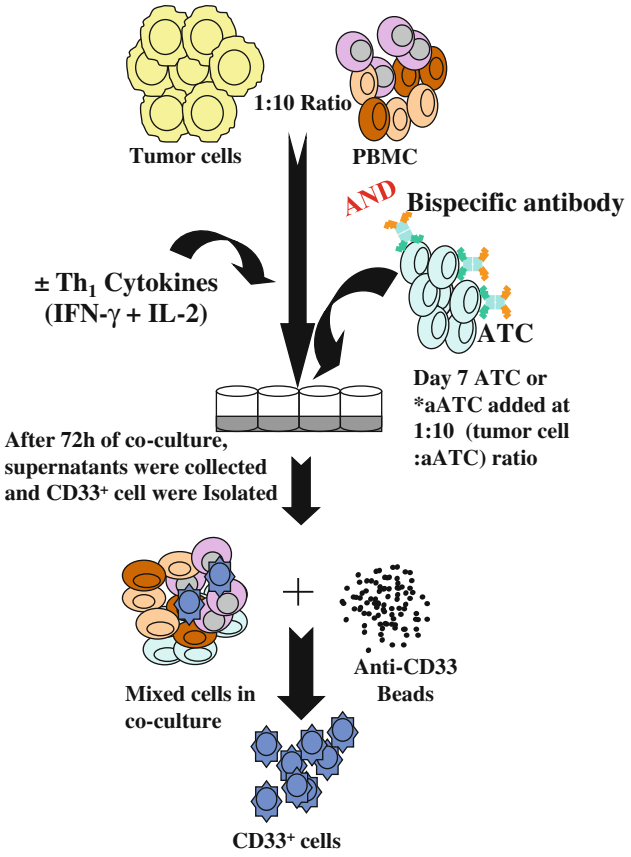


Fig. 1 Co-cultures in 3D model to assess the effects of Th_1 cytokines and Her2Bi-armed ATC (aATC) on the development and regulatory activity of MDSC. PBMC were plated in matrigel at 1:10 (tumor cell/PBMC). aATC were added after a week of tumor cells and PBMC co-culture. Following 3–5 days of additional co-culture, matrigel was digested and single-cell suspension was harvested for phenotyping or separation of $CD33^+$ cells to evaluate their functional properties. Culture supernatants were assessed for cytokine levels

incubation, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) was added (40 µl/well of 5 mg/ml MTT in PBS) to each well and incubated in the dark for 3 h at 37°C. After removal of the medium, the dye crystals formed in viable cells were dissolved in isopropanol and detected by reading the absorption at 595 nm in the Tecan Ultra plate reader. Experiments were repeated three times in quadruplicate wells to ensure the reproducibility of results.

Flow cytometric quantification of $CD11b^+CD33^+$/$HLA\text{-}DR^-$ MDSC and $CD4^+/CD25^+/CD127^{lo}$ regulatory T cells

The phenotype of MDSC generated in 3D co-culture of tumor cells with PBMC was evaluated for the expression of CD33, CD11b, CD14 and HLA-DR. After non-adherent cells were collected, matrigel was digested to collect tumor cells, tumor-associated MDSC or Tregs, washed with FACS buffer (0.2% BSA in PBS). Cells collected prior to digestion were pooled with matrigel-digested single-cell suspension before staining. Cells were stained for 30 min on ice with mixtures of fluorescently conjugated mAbs or isotype-matched controls, washed twice with FACS buffer and analyzed. Antibodies used for staining include: anti-CD3, -CD4, -CD25, -CD127, -CD11b, -CD14, -CD33, and -HLA-DR (BD Biosciences San Jose, CA). Cells were analyzed on a FACScalibur (BD Biosciences), and data were analyzed using CellQuest software (BD Biosciences). Cells were gated for $CD14^-/HLA\text{-}DR^-$ and $CD14^+/HLA\text{-}DR^-$ expression and analyzed for $CD11b^+$ versus $CD33^+$ expression. Tregs were determined by gating for $CD4^+$ expression and analyzed for $CD25^{hi}$ versus $CD127^{lo}$ expression.

Cytokine profiling of co-cultures

Cytokines were quantitated in culture supernatants collected from matrigel co-cultures in the presence or absence of Th_1 cytokines and in the presence or absence of armed ATC using a 25-plex human cytokine Luminex Array (Invitrogen, Carlsbad, CA) on a Bio-Plex system (Bio-Rad Lab., Hercules, CA). The limit of detection for these assays is <10 pg/ml based on detectable signal of greater than twofold above background (Bio-Rad). Cytokine concentrations were automatically calculated by the BioPlex Manager Software (Bio-Rad).

MDSC isolation

Cells were collected from the digested matrigel cultures. $CD33^+$ cells were isolated from each culture using anti-CD33 magnetic microbeads (Miltenyi Biotec,). The purity of isolated cell populations was found to be >90% by flow cytometry.

Inhibition of IFN-γ-secretion by MDSC

Effect of MDSC on IFN-γ production by tumor cell stimulated aATC were detected by IFN-γ-specific EliSpot assay (BD Biosciences, San Jose, CA). Positive control EliSpots were assessed after 18 h exposure to the stimulant cells at 10:1 effector to target ratio (E/T). Effect of MDSC was assessed at 10:2:1 ratio (10 aATC: 2 MDSC: 1 tumor cell) in EliSpot plates [28]. Spots were captured and counted on CTL Immunospot counter using Immunospot software version 4 (Cellular Technology Ltd, Shaker Heights, OH).

Inhibition of T-cell proliferation by MDSC

To measure the inhibitory capacity of the MDSC cells, $CD33^+$ cells isolated from matrigel co-cultures were

co-incubated with purified $CD3^+$ T cells. Briefly, purified CD3 + T cells plated at 0.5×10^4 cells/well in 96-well microtiter plates coated with anti-CD3 antibodies (0.5 μg/ml in PBS), and irradiated (2,500 rads) $CD33^+$ cells were added at various ratios of MDSC/T cells ranging from 1:5 to 1:20 in a final volume of 200 μl of medium. Control wells did not receive any MDSC. The plates were incubated for 72 h at 37°C in humidified 5% CO_2 atmosphere followed by a proliferation assay determined by titer-Glo (Promega).

Inhibition of cytotoxicity by MDSC

MDSC suppression of aATC-mediated cytotoxicity directed at specific targets was measured by chromium (^{51}Cr) release assay in 96-well flat-bottomed microtiter plates as described [25]. Briefly, aATC were added to target cells at 10:1 E/T in the absence or presence of $CD33^+$ cells at 10:2:1 (aATC/MDSC/tumor cell) ratio. ^{51}Cr release was measured after 18 h, and percent cytotoxicity was calculated using the following formula: (experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm) ×100.

Statistical analysis

Quantitative data are presented as the mean of at least three or more independent experiments ± standard deviation. A one-way ANOVA was used to determine whether there were statistically significant differences within each experiment. Differences between groups were tested via an unpaired, two-tailed *t* test.

Results

Th_1 cytokine–enriched microenvironment inhibits tumor growth

First, we explored whether Th_1 cytokine–enriched microenvironment can suppress tumor growth. To this end, we set up the matrigel (3D) cultures of BrCa (SK-BR-3 and MDA-MB-231) cell lines in the presence or absence of IFN-γ (10 ng/ml) and IL-2 (100 IU/ml). SK-BR-3 cells over-expresses Her2/*neu* with epitheloid morphology, whereas MDA-MB-231 is a triple-negative and highly invasive cell line with mesenchymal morphology. Both BrCa cell lines grown under Th_1 cytokine–enriched conditions exhibited smaller tumor spheres in 3D culture as compared to larger tumor spheres in regular growth medium without Th_1 cytokines (Fig. 2a, top at 20× and bottom panel at 100× magnification).

Th_1 cytokine–enriched microenvironment enhances aATC-mediated tumor cell killing

Next, we tested whether the Th_1 cytokine–enriched microenvironment promoted aATC-mediated killing of tumor spheres. This was tested using visual inspection by confocal imaging in 3D cultures and by the MTT assay in 2D cultures. For confocal imaging, BrCa cells were labeled with DiI (red) at day 7 followed by co-culture with DiO (green)-labeled aATC at a 10:1 E/T for an additional 72 h. Confocal imaging show that aATC completely surrounded the tumor spheres, suggesting highly efficient binding of aATC with tumor cells in the presence or absence of Th_1 cytokines. In the presence of Th_1 cytokines, aATC are able to kill tumor spheres effectively as evidenced by a marked decrease in tumor spheres (Fig. 2b). Figure 2b shows confocal imaging of SK-BR3 (upper panels) and MDA-MB-231(lower panels). These results show that Th_1 cytokines inhibit tumor growth and prime tumor cells for enhanced killing by aATC. Cytotoxicity quantitated by MTT assay in 2D cultures of tumor cells and aATC for 3–5 days at 1:1 (Fig. 2c) E/T in the presence or absence of Th_1 cytokines corroborate the results of confocal imaging that Th_1 cytokines render susceptibility to tumor cells for aATC ($n = 3$; $P < 0.001$)-mediated killing (Fig. 2c).

Th_1 cytokine–enriched microenvironment inhibits MDSC development

Since IFN-γ has been reported to have conflicting effects on MDSC development, we asked whether a Th_1 cytokine–enriched microenvironment can modulate MDSC development. A representative flow cytometric analysis of MDSC populations presented in Fig. 3a clearly shows a reduced percentage of $CD33^+/CD11b^+/CD14^-/HLA\text{-}DR^-$ MDSC population in the presence of Th_1 cytokines for both cell lines. Quantitative data in Fig. 3b show that $CD33^+/CD11b^+/CD14^-/HLA\text{-}DR^-$ MDSC population is significantly reduced for both cell lines (SK-BR-3 co-cultures, $P < 0.013$; for MB-231 co-cultures, $P < 0.037$) in the presence of Th_1 cytokines compared to co-culture without Th_1 cytokines. Percentage of $CD33^+/CD11b^+/CD14^+/HLA\text{-}DR^-$ population slightly increased in the presence of Th_1 cytokines compared to co-cultures without Th_1 cytokines, but the difference was not significant ($n = 3$). These data suggest that Th_1 cytokine–enriched microenvironment inhibits the development of MDSC. No difference was observed for the Treg $CD4^+/CD25^{hi}/CD127^{lo}$ population in the presence or absence of Th_1 cytokines for both cell lines (Fig. 3a, b, bottom panels).

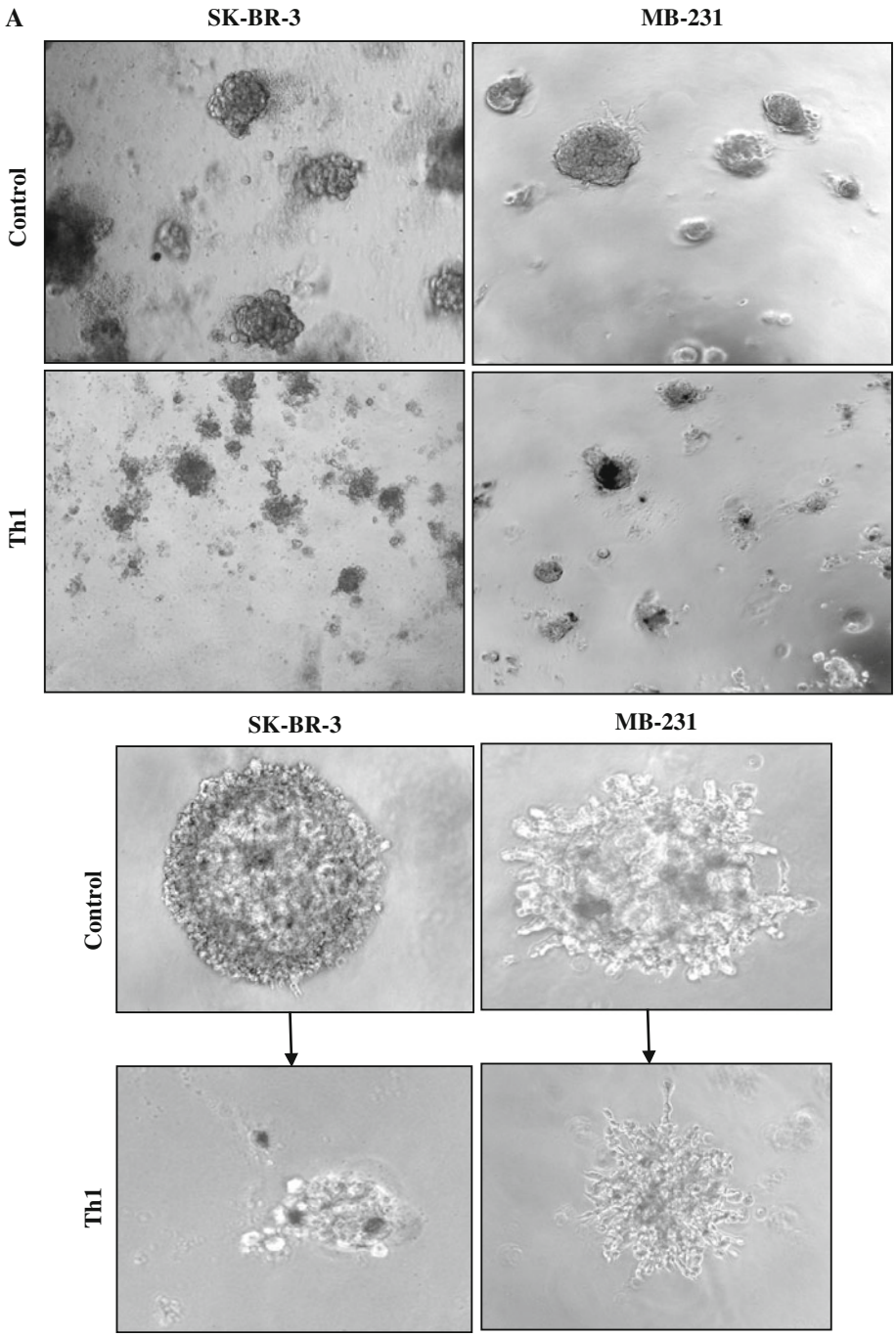


Fig. 2 **a** Shows the effect of Th_1 cytokines on tumor growth at day 7 using inverted microscopy. Visibly reduced size of tumor spheres for SK-BR-3 and MB-231 BrCa cells were evident under Th_1 cytokines when compared with control condition (*top panels*, 20×; *bottom panels*, 100× magnification). **b** Confocal imaging of BrCa cells labeled with DiI (*red fluorescent dye*) at day 7 followed by co-culture with DiO (*green fluorescent dye*)-labeled aATC at a 10:1 E/T for an additional 72 h shows that armed ATC was able to engage tumor cells. In the presence of Th_1 cytokines, reduced size of tumor spheres were observed (20× magnification). **c** Cytotoxicity quantitated by MTT assay in 2D cultures of tumor cells and aATC for 3 days at an E/T ratio of 1:1

Her2Bi-armed activated T cells (aATC) inhibit the development of MDSC and Tregs under both control and Th_1 cytokine–enriched conditions

Because aATC-mediated killing of tumor cells produce Th_1 cytokines and chemokines, we hypothesized that microenvironment generated during aATC interaction with tumor cells modulate the function or development of MDSC. A representative flow analysis of MDSC and Treg populations is shown in the presence of aATC under both control and Th_1 cytokine–enriched culture conditions for SK-BR-3 and MB-231 in Fig. 3c (upper and lower panels). Reduction in $CD33^+/CD11b^+/CD14^-/HLA\text{-}DR^-$ MDSC population was highly significant under both control (SK-BR-3, $P < 0.0001$; MB-231, $P < 0.03$) and Th1 (SK-BR-3, $P < 0.001$; MB-231, $P < 0.036$) conditions in the presence of aATC compared to without aATC (Fig. 3d). These data suggest that aATC-generated microenvironment leads to MDSC elimination and is not dependent on pre-existing Th_1-enriched microenvironment ($n = 3$). Unlike MDSC, a significant suppression of $CD4^+/CD25^{hi}/CD127^{lo}$ Treg differentiation ($P < 0.028$) only occurred in the presence of both a Th_1

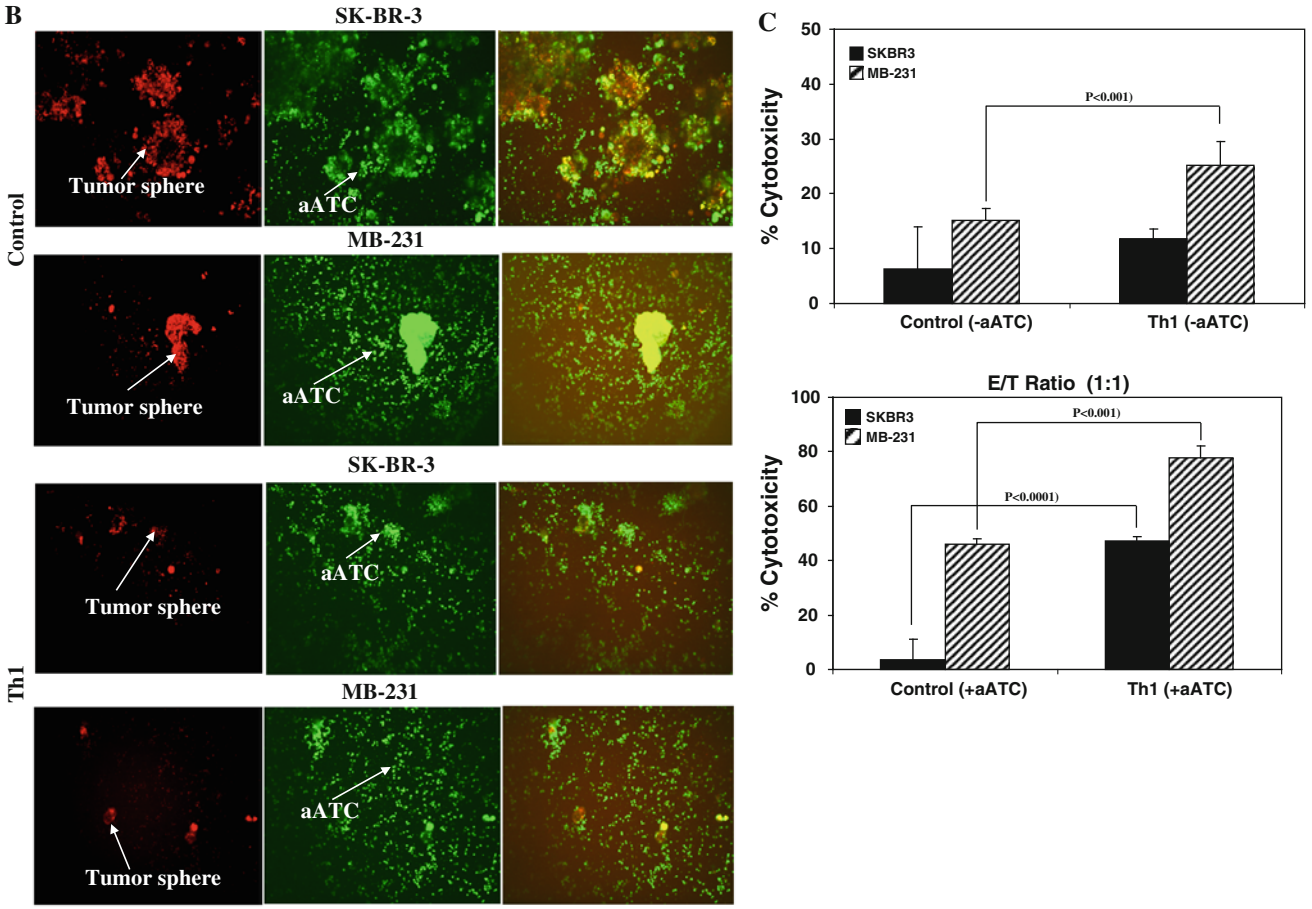


Fig. 2 continued

cytokines–enriched microenvironment and aATC (Fig. 3c, d, lower panels) for SK-BR-3 cells. No difference was observed for MB-231 cultures with or without Th_1 cytokines and aATC.

Comparison of MDSC populations in culture conditions with or without aATC showed significantly reduced percentage of $CD33^+/CD11b^+/CD14^-/HLA\text{-}DR^-$ MDSC population in aATC-containing control culture ($P < 0.001$) or aATC-containing Th_1-enriched culture condition ($P < 0.001$) compared with control or Th_1-enriched culture conditions without aATC. However, no difference was observed for $CD33^+/CD11b^+/CD14^+/HLA\text{-}DR^-$ MDSC population among these culture conditions.

Her2Bi-armed activated T cells (aATC) attenuates the immunosuppressive ability of MDSC. Next, we tested whether MDSC can exhibit an inhibitory effect on T-cell functions and whether presence of Th_1 cytokines and aATC can modulate the suppressive activity of MDSC.

Suppression of proliferation

For this, cells were sorted into $CD33^+$ MDSC and co-cultured with aATC at 1:5 ratio. T-cell proliferation was suppressed by 70–90% with the addition of $CD33^+$ MDSC; noteworthy is that cultures containing aATC reversed the suppressive ability of $CD33^+$ MDSC by >50% in the presence or absence of Th_1 cytokines for both cell lines ($n = 3$; Fig. 4, top panel).

Suppression of cytotoxicity

Furthermore, we asked whether the addition of MDSC to aATC would inhibit the ability of Her2Bi-armed ATC to kill SK-BR-3 targets at a 1:10:2 ratio (Tumor cell/aATC/$CD33^+$). The cytotoxicity mediated by aATC directed at SK-BR-3 targets was inhibited by 75% in the presence of $CD33^+$ cells. Interestingly, $CD33^+$ cells isolated from aATC-containing co-cultures showed a significant attenuation in suppressive ability of $CD33^+$ MDSC for both BrCa cell lines ($n = 3$; Fig. 4, middle panel).

Suppression of IFN-γ production by aATC

$CD33^+$ MDSC also suppressed IFN-γ production in target stimulated aATC by 70–75%. The addition of aATC to the co-cultures attenuated the suppressive ability of $CD33^+$ MDSC by ~ 50–60% for both BrCa cell lines ($n = 3$; Fig. 4, bottom panel).

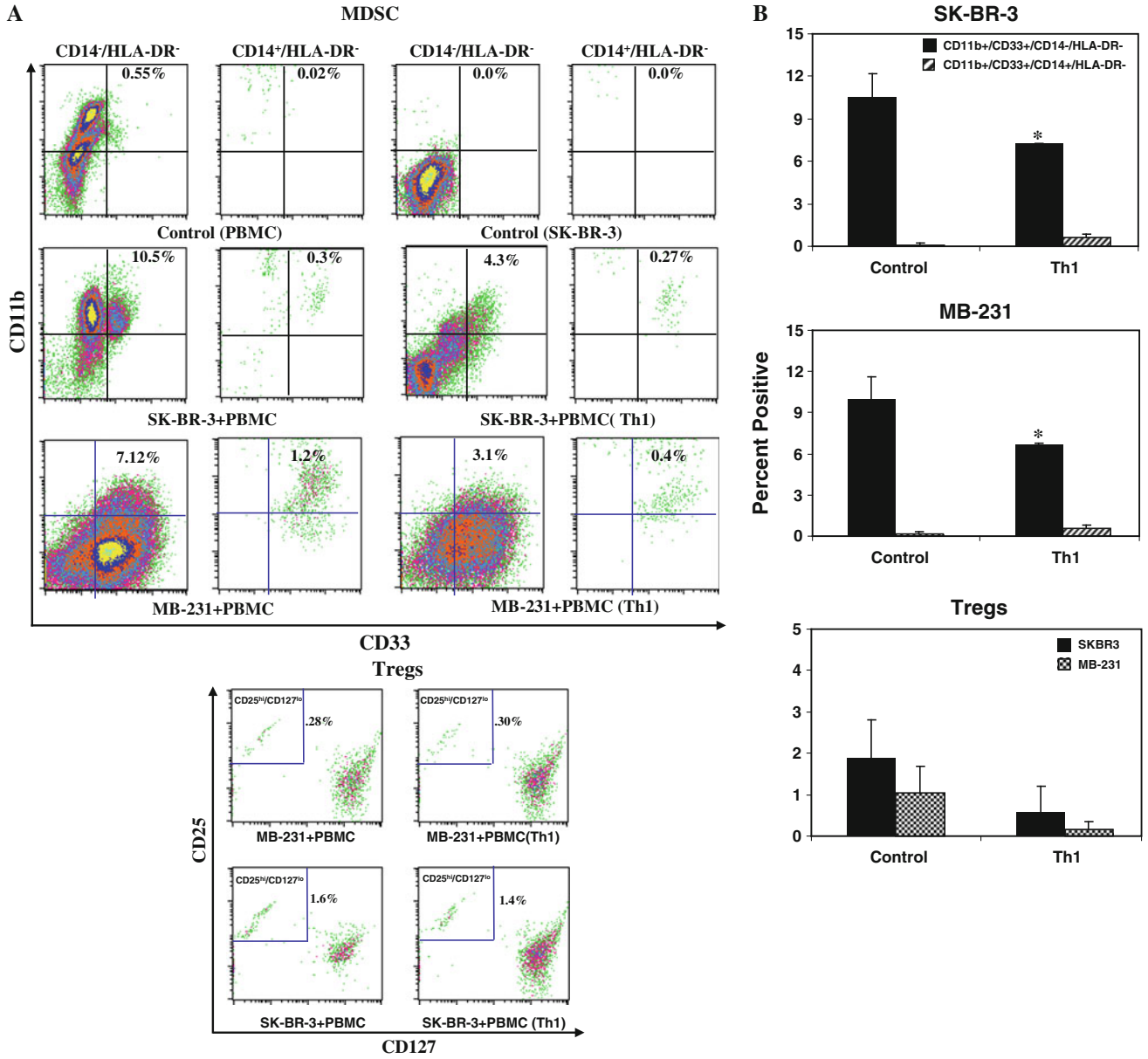


Fig. 3 Shows a representative flow cytometry data for MDSC and T_{reg} populations in the presence or absence of Th_1 cytokines and aATC for SK-BR-3 and MB-231 co-cultures. **a** Flow cytometry showing the MDSC populations, $CD11b^+$ and $CD33^+$ population was gated on either $CD14^-$/HLA-DR^- (granulocytic) or $CD14^+$/HLA-DR^- (monocytic) cell populations. The granulocytic population was reduced by less than half in the presence of Th_1 cytokines. The lower panel shows the flow cytometry data for T_{reg} cells in the presence or absence of Th_1 cytokines, $CD25^{hi}$ and $CD127^{lo}$ population was gated on $CD4^+$ cells. **b** Shows the quantitative data ($n = 3$) for MDSC (*upper* and *middle panels*) and T_{reg} populations under control and Th_1 culture conditions (*lower panel*). **c** Shows the representative flow cytometry data for MDSC for SK-BR-3 (*upper panel*, *top*) and for MB-231 (*upper panel*, *bottom*) and Treg (*lower panel*) populations in the presence or absence of Th_1 cytokines and aATC. Both MDSC and T_{regs} were reduced in the presence of Th_1 cytokines and/or aATC. **d** Shows the quantitative data ($n = 3$) for MDSC and T_{reg} populations in the presence or absence of Th_1 cytokines and aATC for both cell lines. */**signifies statistically significant differences between control and Th_1-enriched culture conditions in the presence or absence of aATC (*$P < 0.05$, **$P < 0.01$)

Low levels of IFN-γ, CXCL9 and CXCL10 were associated with increased numbers of MDSC

We examined whether the presence of MDSC in co-cultures will induce cytokines/chemokines that promote expansion and activation of MDSC and whether Th_1 cytokine–enriched microenvironment can alter the cytokines/chemokines induced by MDSC. Our data show marked increases in the proinflammatory cytokines IL-1β and IL-6 in control culture supernatants compared with Th_1

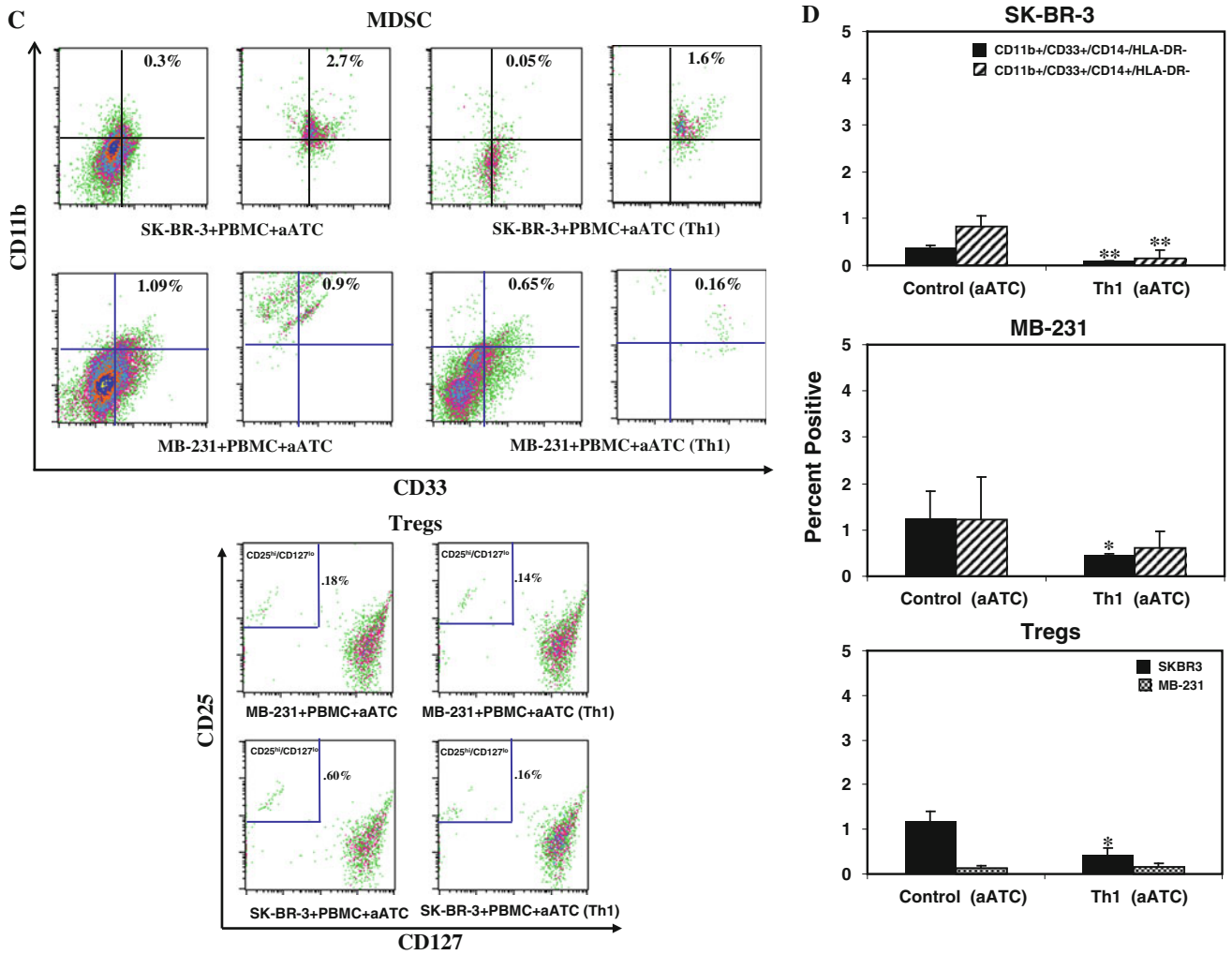


Fig. 3 continued

cytokines–enriched culture supernatants for both SK-BR-3 and MB-231 cell lines (Fig. 5a). On the other hand, IFN-γ and chemokines induced by IFN-γ MIG/CXCL9 and IP-10/CXCL10 were present in extremely low levels in control culture supernatants compared with Th_1 cytokines–enriched culture supernatants for both SK-BR-3 and MB-231 cell lines. Pattern for MCP-1/CCL2 expression was different in both cell lines. In MB-231, high levels of MCP-1/CCL2 were present in both Th_1 cytokines–enriched culture supernatants and control culture supernatants. Whereas, in SK-BR-3, MCP-1/CCL2 protein was not detected in Th_1 cytokine–enriched culture supernatants compared high levels in control culture supernatants. In Th_1 cytokine–enriched microenvironment, MIG/CXCL9 and IP-10/CXCL10 were upregulated, while IL-1β and IL-6 were downregulated with concurrent reduction in the percentage of MDSC compared with control condition. These data suggest that low levels of IFN-γ, MIG/CXCL9 and IP-10/CXCL10 along with high levels of IL-1β and IL-6 may promote MDSC differentiation.

Increased levels of IFN-γ, CXCL9 and CXCL10 corroborate with reduced number of MDSC

Next, we addressed whether the presence of aATC can influence the cytokine and chemokine profiles. Proinflammatory cytokines IL-1β and IL-6 levels were not altered in the presence of aATC under control and Th_1 cytokine–enriched culture conditions compared to without aATC (Fig. 5a). However, in the presence of aATC MIG/CXCL9 and IP-10/CXCL10 were highly upregulated under both control and Th_1 culture conditions (Fig. 5b). These data suggest that high levels of IFN-γ-induced CXCL9 and CXCL10 may contribute in the suppression MDSC development. To confirm whether increased levels of MIG/CXCL9 and IP-10/CXCL10 may suppress MDSC

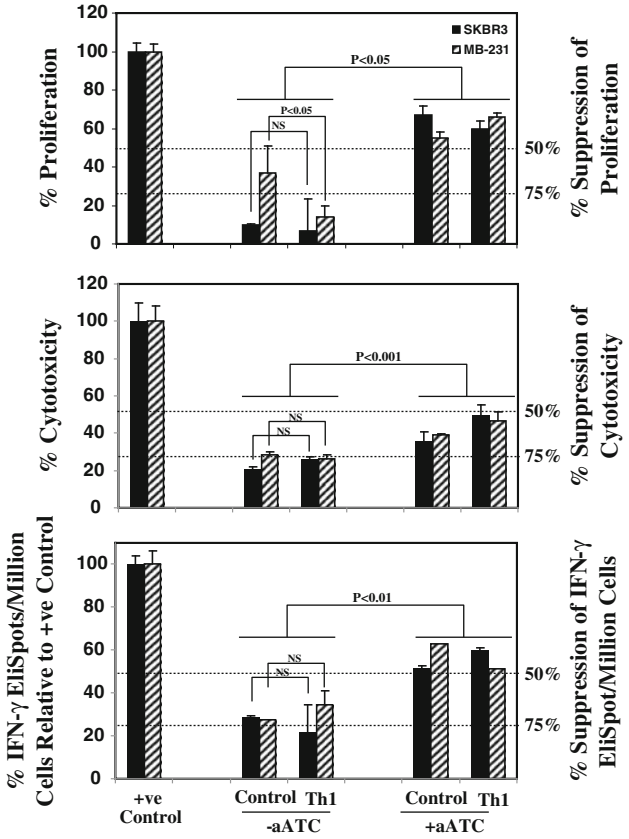


Fig. 4 Functional properties of MDSC. For analysis of inhibitory ability of MDSCs, $CD33^+$ cells were added at 1:5 ($CD33^+$/aATC) ratio to T-cell proliferation, 1:2 ratio to cytotoxicity and IFN-γ EliSpot assays. *Top panel* shows the suppressive effect of $CD33^+$ MDSC on anti-CD3-stimulated autologous T-cell proliferation. Proliferation was significantly suppressed by 70–90% in the presence of $CD33^+$ MDSC that was reversed if aATC were present in co-cultures. $CD33^+$ cells isolated from various culture conditions when added to positive controls (Her2Bi-armed ATC-mediated killing of SK-BR-3 for cytotoxicity; SK-BR-3 stimulated Her2Bi-armed ATC for EliSpots) showed approximately 75% suppression of cytotoxicity (*middle panel*) and IFN-γ EliSpots (*bottom panel*). In the presence of aATC under both control and Th_1-enriched culture conditions, suppressive ability of $CD33^+$ cells was significantly attenuated

development, we added these two chemokines in control culture condition in the presence or absence of aATC. Intriguingly, co-cultures containing MIG/CXCL9 and IP-10/CXCL10 in the absence of aATC showed reduced percentage of $CD33^+/CD11b^+/CD14^-/HLA\text{-}DR^-$ MDSC population (Fig. 5c), suggesting that MIG/CXCL9 and IP-10/CXCL10 may suppress MDSC development.

Discussion

This is the first study to show that a Th_1 cytokine–enriched microenvironment hampered tumor growth, significantly enhanced aATC-mediated killing of tumor cells and significantly reduced the absolute numbers of granulocytic $CD14^-/HLA\text{-}DR^-/CD11b^+/CD33^+$ and monocytic $CD14^+/HLA\text{-}DR^-/CD11b^+/CD33^+$ MDSC populations compared with control culture conditions. More importantly, co-cultures with aATC showed highly significant reduction in MDSC populations regardless of Th_1 cytokines in culture. Furthermore, $CD33^+$ MDSC isolated from cultures containing aATC exhibited reduced suppressive ability, suggesting that aATC were able to attenuate the suppressive activity of MDSC.

In order to understand how Th_1 cytokines and aATC affect the development of MDSC, we analyzed the cytokine profiles in the culture supernatants produced by cells in co-culture under both Th_1 and control conditions with or without aATC. Our data show distinct patterns of cytokines and chemokines in Th_1 cytokine–enriched versus control culture conditions. There were lower levels of cytokines IL-6 ($\sim$ threefold lower) and IL-1β under Th_1 cytokines–enriched culture supernatants (contained artificially high IFN-γ) compared with control culture supernatants for both cell lines. Cytokines provide key signaling in the generation of MDSC; both IL-6 and IL-1β have been shown to promote in vitro generation and the regulation of MDSC and their suppressive function in vivo [23–25]. Consistent with these reports, our results show decreased levels of IL-6 and IL-1β associated with decreased numbers of MDSC in Th_1 cytokines–enriched culture supernatants. On the other hand, increased levels of IL-6 and IL-1β in control culture supernatants were associated with increased numbers of MDSC underscoring their roles in generation and/or activation of MDSC.

Similar to cytokines, chemokines exhibited different expression patterns between control and Th_1 cytokine–enriched culture conditions. Our data show higher levels of IFN-γ-induced CXC chemokines such as MIG/CXCL9 (monokine induced by IFN-γ) and IP-10/CXCL10 (interferon-inducible protein-10) under Th_1 condition. The higher levels of IFN-γ in both cell lines were associated with decreased numbers of MDSC in co-cultures containing Th_1 cytokines. Both, SK-BR-3 and MB-231, cells showed high levels of MCP-1/CCL2 expression under control condition, but levels were reduced under Th_1 condition for MB-231 cells, while MCP-1/CCL2 protein was not detectable for SK-BR-3 in Th_1 cytokines–enriched culture supernatants. MIG/CXCL9 and IP-10/CXCL10) are chemoattractants for IL-2 inducible CXCR3-expressing activated T cells [26, 27] and inhibit the neovascularization induced by powerful angiogenic factors such as MCP-1/CCL2 through CXCR3 receptor signaling [28]. These CXC chemokines with their ability to recruit T cells and inhibit angiogenic activity, suggest these chemokines as potential anti-tumor factors. However it is not known how these chemokines affect MDSC differentiation in our in vitro 3D

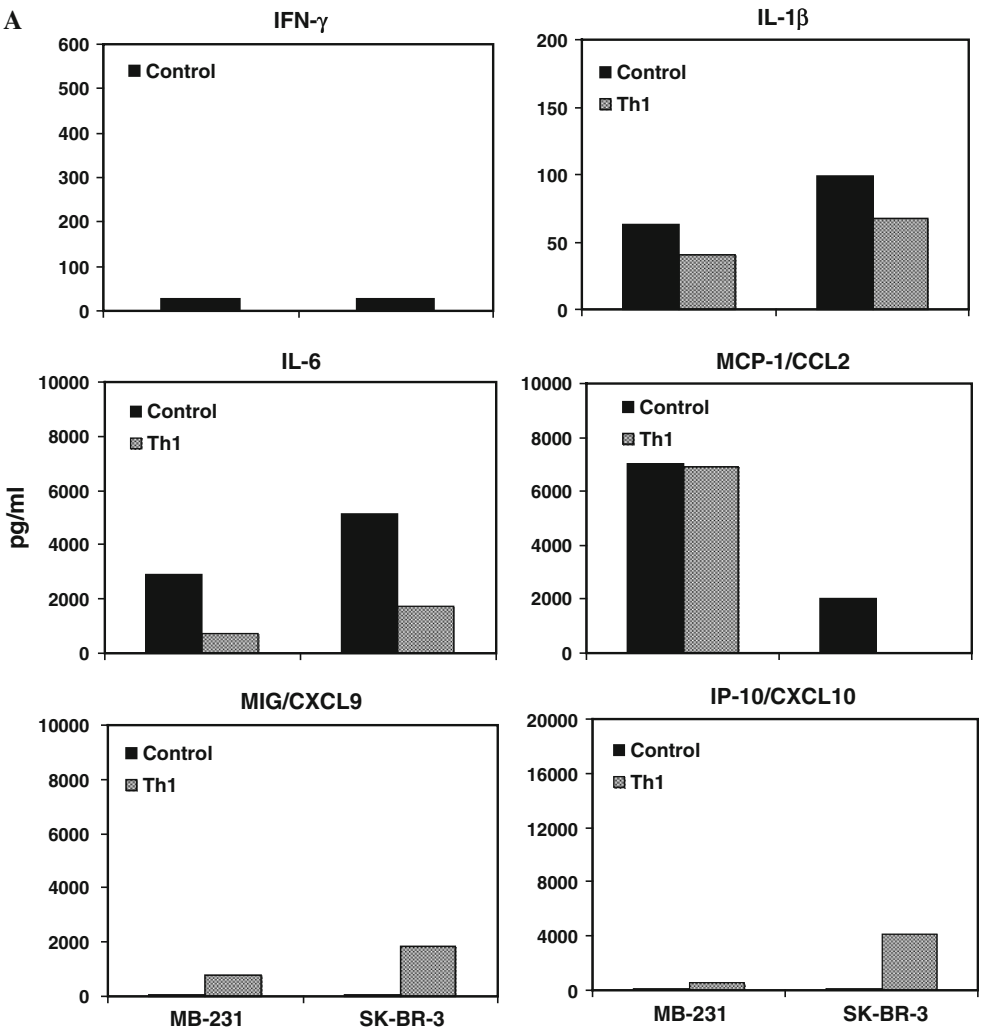


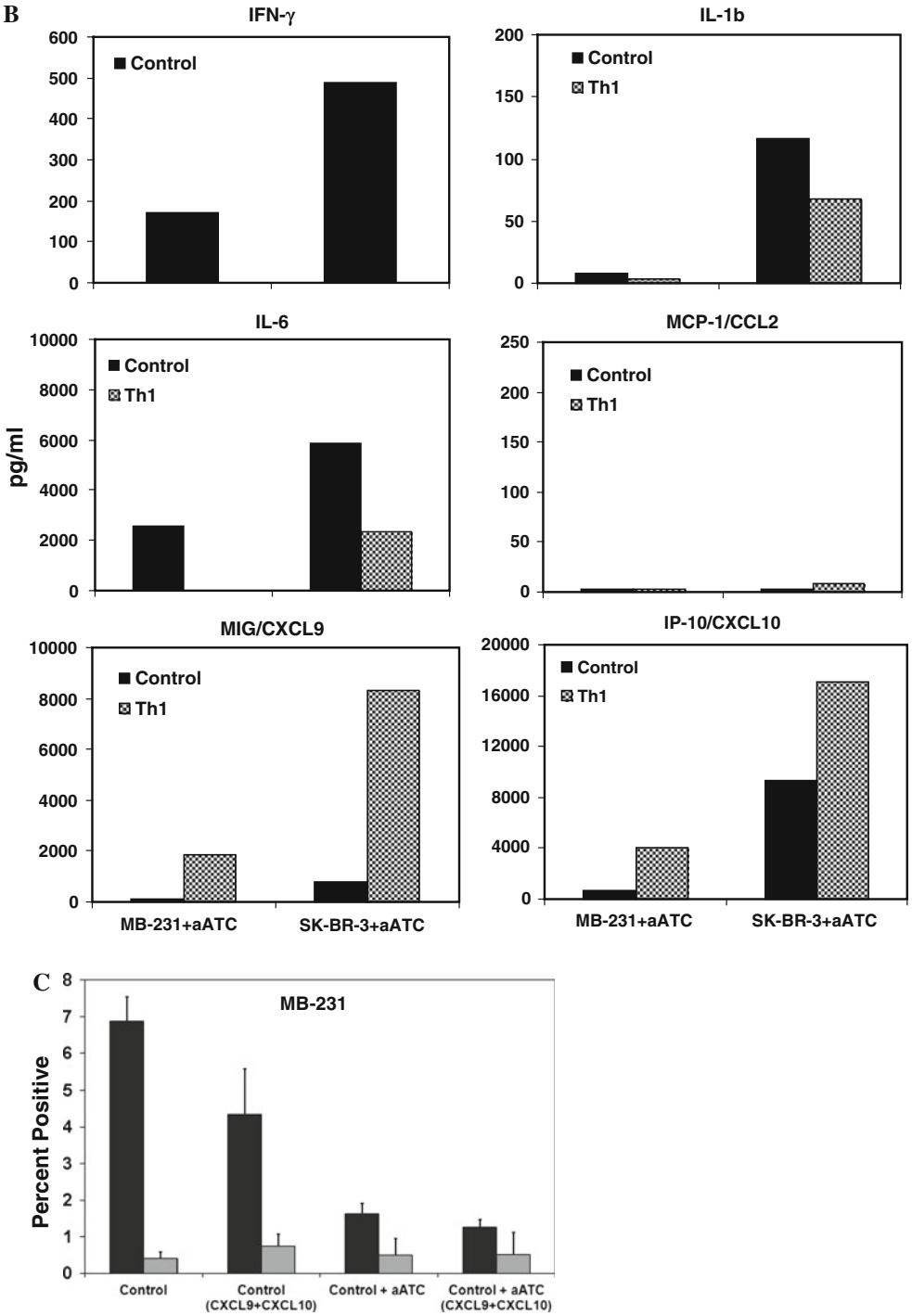
Fig. 5 Cytokine profiles of culture supernatants detected by multiplex luminex system show the increased levels of cytokines IL-1β and IL-6, and chemokine MCP-1 in the absence of Th_1 cytokines, while IFN-γ inducible chemokines MIG/CXCL9 and IP-10/CXCL10 showed increased levels in the presence of Th_1 cytokines **a** in the presence or absence of Th_1 cytokines and **b** in the presence of aATC under control and Th_1 cytokines, **c** Shows significantly reduced percentage ($P < 0.05$) of $CD33^+/CD11b^+/CD14^-/HLA\text{-}DR^-$ MDSC population in the presence of recombinant human CXCL10 and CXCL9 proteins in control culture condition

model, we hypothesize that these chemokines may potentiate the anti-tumor activity of T cells directly affecting tumor growth which in turn can affect MDSC development and activation.

Since aATC can produce IFN-γ upon interaction with tumor targets, we hypothesized that presence of aATC in control cultures (resembling the in vivo aATC immunotherapy situation) will induce high levels of IFN-γ, CXCL10 and CXCL9 and decrease in MDSC populations. As predicted, control cultures containing aATC showed the similar pattern of cytokines and chemokines as was in Th_1 cytokine–enriched culture condition without aATC. In addition, aATC in control co-cultures downregulated MCP-1/CCL2. Although the pattern of all the cytokines and chemokines was similar to Th_1 cytokines–enriched culture condition except for MCP-1/CCL2, the levels were strikingly lower in cultures containing aATC under both culture conditions. MCP-1/CCL2 is an angiogenic and immunomodulatory factor that is highly chemotactic for monocytes and regulatory T cells [29, 30] while specifically inhibiting $CD8^+$ T-cell effector functions [31]. Huang et al. [32]. showed that the recruitment of MDSC into tumors is mediated by the CCL2/CCR2 axis and absence of CCL2/CCR2 signaling hindered both MDSC migration and MDSC-promoted tumor growth. In our in vitro 3D model, lower levels of MCP-1/CCL2 may have added effect in suppressing MDSC development in addition to suppressive affects of increased levels of IFN-γ, CXCL10 and CXCL9 on MDSC. The role of CXCL10 and CXCL9 in suppressing MDSC population was further evidenced by adding CXCL10 and CXCL9 in control culture condition that resulted in reduced percentage of MDSC (Fig. 5c).

Chemokines can play dual role in tumor development [33]. Mullins et al. [34, 35]. reported that recruitment of CD8 + T lymphocytes expressing CXCR3 by chemokines leads to the improvement of patient survival in melanoma. Lack of critical chemokines (CCL2, CXCL9 and CXCL10) in melanoma metastases aggravated disease due to blockage in activated T-cell migration and anti-tumor immunity [36]. On the other hand, the aberrant expression of

Fig. 5 continued



chemokines in tumors has been shown to induce immunosuppression that favors tumor growth. In hepatocellular carcinoma, high levels of CXCL9 and CXL10 have been associated with inhibition of CXCR3 expression by CD8 + T lymphocytes, reduction in T-cell tumor infiltration and cytotoxic functions and tumor growth [37].

The important finding in this study is that IFN-γ was able to suppress both tumor growth and MDSC development and rendering tumor cells susceptible to enhanced aATC-mediated killing. However, the mechanisms of multiple effects of IFN-γ in our model are not clear, and thus warrants further investigation. While IFN-γ has been shown to sensitize tumor cells for enhanced Fas-mediated killing by CTL [38, 39], role of IFN-γ in the development and activation of MDSC remains controversial. However, a recent study demonstrated that activated T cells can mediate MDSC apoptosis through Fas/FasL pathway [40]. IFN-γ produced by tumor-specific T lymphocytes has been

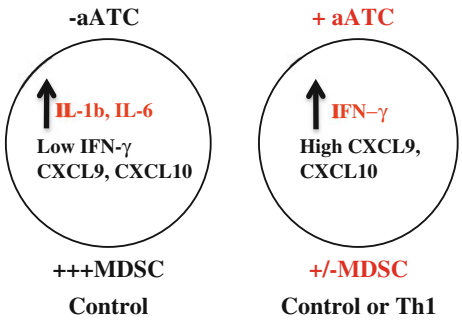


Fig. 6 A schematic summary of the present study show that high levels of IFN-γ either in the presence of aATC alone under control condition or in the presence of aATC and Th_1 cytokines can induce high levels of IFN-γ-driven chemokines CXCL9 and CXCL10 that may suppress MDSC development and differentiation. On the other hand, in the control condition, high levels of tumor-derived IL-1β and IL-6 may negate the effects of low levels of IFN-γ and IFN-γ-driven chemokines CXCL9 and CXCL10 and may support MDSC development

shown to trigger MDSC maturation with subsequent amplification of IL-13 and IFN-γ produced by MDSC to maintain a prolonged activation of the immunosuppressive mediators NOS and ARG [8]. On the contrary, Nonaka et al. showed that Th_1-dominant tumor environment induced marked T-cell infiltration, created IFN-γ- and GM-CSF-rich microenvironment without IL-4 or IL-13. The absence of IL-4 and IL-13 in the Th_1-dominant and T-cell infiltration-rich environment suppressed the maturation of MDSC compared with IL-4 and IL-13 (Th_2-enriched microenvironment) [41]. Based on our data, we propose a working hypothesis that context-dependent homeostatic balance of various cytokines and chemokines may determine their pro or anti-MDSC activity (Fig. 6).

In summary, we show that either an artificially generated Th_1 cytokine–enriched microenvironment or Th_1 microenvironment generated during aATC-mediated killing of tumor cells, both conditions resulted in a significant reduction in MDSC development or elimination, respectively. Our study suggests that targeted immunotherapy with aATC directed at tumor targets will not only kill tumor cells but may also reduce MDSC populations as part of the targeting response. Further studies are needed to demonstrate the effect of aATC therapy on MDSC in pre- and post-aATC-treated breast cancer patients.

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Conflict of interest L.G.L. hold founder's shares of Transtarget, Inc. The other authors have no financial conflict of interest.

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