

IL-2 Costimulation Enables Statin-Mediated Activation of Human NK Cells, Preferentially through a Mechanism Involving CD56⁺ Dendritic Cells

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Abstract

Statins are inhibitors of cholesterol biosynthesis and protein prenylation that also have been studied in cancer therapy and chemoprevention. With regard to natural killer (NK) cells, only inhibitory effects of statins such as suppression of granule exocytosis have been reported so far. In this study, we show that statins can cooperate with IL-2 to potentially induce the activation of CD56^{dim} NK cells in a synergistic, time- and dose-dependent fashion. Supplementation experiments revealed that the statin effect was specific to inhibition of their target hydroxymethylglutaryl coenzyme A reductase and that downstream depletion of geranylgeranyl pyrophosphate was responsible for cooperating with IL-2 in NK cell activation. Mechanistic studies revealed that CD56⁺HLA-DR⁺CD14⁺ dendritic cell (DC)-like accessory cells mediated the ability of statin to activate NK cells. In contrast, BDCA-1⁺ (CD1c⁺) myeloid DCs, which partially expressed CD56, were somewhat less potent. Conventional blood monocytes, which lack CD56, exhibited the lowest accessory cell capacity. NK cell IFN- γ production was IL-12 independent but required endogenous IL-18, IL-1 β , and caspase-1 activity. Statins directly induced apoptosis in human cancer cell lines and cooperated with NK cell-derived IFN- γ to generate potent cytotoxic antitumor effects *in vitro* even in the presence of statin-mediated inhibitory effects on granule exocytosis. Our work reveals novel and unexpected immunomodulatory properties of statins, which might be harnessed for the treatment of cancer. *Cancer Res*; 70(23); 9611–20. ©2010 AACR.

Introduction

Statins, which are primarily used to control hypercholesterolemia, have also been proposed as anticancer agents (1, 2). They inhibit hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the first committed step of the mevalonate pathway. Mevalonate, the immediate product of HMG-CoA, is further metabolized to form important cellular products such as cholesterol or farnesyl pyrophosphate (FPP) as well as geranylgeranyl pyrophosphate (GGPP; refs. 3, 4), which are required for protein prenylation (3, 4). Prenylation frequently occurs on small GTPases of the RAS superfamily (2).

Statin-mediated inhibition of mevalonate metabolism affects many downstream pathways and may thus influence

cell membrane integrity, steroid content, cell signaling, and respiration, as well as inflammatory and immune responses (1, 2). Because these pathways are all critical for cancer development and progression, statin-mediated inhibition of these processes is believed to inhibit tumor initiation, growth, and metastasis.

Modulation of protein prenylation is considered a key mechanism, by which statins alter inflammatory and immune responses (4). Although it is well established that statins are predominantly anti-inflammatory and immunosuppressive agents, an increasing number of studies have shown that statins may also induce proinflammatory responses depending on costimulatory signals (5–8). In contrast, with regard to NK cells, only inhibitory effects have been reported so far (9–12). However, all these studies focused on NK cell–target cell interactions but did not investigate the role of antigen-presenting cells (APC) in statin-mediated NK cell modulation.

We and others have previously detected an HLA-DR⁺CD14⁺ cell subset in human blood that expresses the CD56 antigen (= neural cell adhesion molecule; refs. 13–15), which is constitutively expressed by NK cells and may be induced by some T-cell subsets during activation (16, 17). These monocyte/dendritic cell (DC)-like cells have been shown to be phenotypically and functionally similar to conventional DCs (13–15).

In the present work, we report, for what we believe to be the first time, that statins not only have direct antitumor effects but may also induce the activation of human NK cells via an

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intriguing subset of CD56⁺ DC-like cells to increase their antitumor efficacy.

Materials and Methods

Reagents, antibodies, and cell lines

Mevastatin, simvastatin, and mevalonolactone were obtained from Sigma-Aldrich. Mevastatin, which is isolated from *Penicillium citrinum*, was the founding member of the statin family. It is not used in therapy but serves as a precursor in the synthesis of other statins. In contrast, simvastatin (Zocor and others), which is a synthetic derivative of a fermentation product of *Aspergillus terreus*, is a widely prescribed statin (Supplementary Fig. S1; ref. 18).

FPP and GGPP were purchased from Echelon Biosciences. Squalene synthase inhibitor YM-53601 was obtained from Cayman Chemical. Recombinant human IL-2 (Proleukin) was from Novartis. Recombinant IL-12 and IL-18 were obtained from Bender MedSystems. Anti-CD3 (SK7-PerCP-Cy5.5), anti-CD14 (MP9-FITC, MP9-APC), anti-CD54 (HA58-PE), anti-CD56 (NKH-1-PE), anti-CD86 (FUN-1-FITC), anti-HLA-ABC (G46-2.6-FITC), and anti-HLA-DR (T36-PE) were all from BD Biosciences; anti-Slan (M-DC8; DD-1-APC) was from Miltenyi Biotec; neutralizing anti-IL-18 (125-2H) was from MBL; neutralizing anti-IL-1 β (8516) and anti-IL-12 (24910) were from R&D; neutralizing anti-IFN- γ (25718.11) was from Sigma-Aldrich; caspase-1 inhibitor Ac-Tyr-Val-Ala-Asp-2,6-dimethylbenzoyloxymethyl-ketone (YVAD) was purchased from Alexis Biochemicals.

A-498, MCF-7, DU-145, and LNCaP represent well-studied examples of kidney (A-498), breast (MCF-7), and prostate cancer (DU-145, LNCaP), respectively. A-498 was obtained from the DSMZ (19–21). MCF-7 (22, 23) was kindly provided by Martin Wurm, Department of Internal Medicine, Innsbruck Medical University. DU-145 (24) and LNCaP (25, 26) were kindly provided by Helmut Klocker, Department of Urology, Innsbruck Medical University. All cell lines were cultured in complete BioWhittaker RPMI 1640 (Lonza) supplemented with 10% FCS (HyClone). LNCaP culture medium was further supplemented with glucose (2.5%).

Cell isolation and depletion

All donors gave written informed consent in accordance with the Declaration of Helsinki to the use of their residual buffy coats for research purposes, with approval from the University Hospital of Innsbruck Review Board. CD56⁺ cells were purified from peripheral blood mononuclear cells (PBMC), using CD56 microbeads and LS columns. In some experiments, PBMCs or CD56⁺ PBMCs were depleted of CD14⁺ cells, using CD14 microbeads and LD columns. In other experiments, PBMCs or CD14⁺ PBMCs were depleted of CD56⁺ cells. All procedures were conducted according to the manufacturer's instructions (Miltenyi Biotec).

Phenotypic analyses

For phenotyping, freshly isolated or cultured cells [(1–3) \times 10⁵] were stained with specific fluorochrome-conjugated antibodies in PBS containing 0.5% FCS and 50 μ g/mL of human

IgG (Octapharma) to block Fc γ receptors. After a 30-minute incubation on ice followed by 2 washes, cells were analyzed using a FACSCanto II system and FACSDiva 6.1.2 and FlowJo V7.2.5 software (BD Biosciences).

Cell culture and stimulation

All cell cultures were conducted in complete BioWhittaker RPMI 1640 (Lonza) supplemented with 10% FCS (HyClone). For cytokine measurements, cells (1 \times 10⁶/mL) were stimulated with IL-2 (100 U/mL) either alone or in combination with statin (0.1–5 μ mol/L) in 96-well round-bottomed plates (0.2 mL). After 1 to 3 days, supernatants were harvested for cytokine analysis. For neutralizing and blocking assays, cultures were preincubated with specific antibodies or inhibitors. Cellular responses were also documented with an Olympus CK2 microscope equipped with a ProgRes CT3 digital camera and ProgRes CapturePro 2.5 software (Jenoptik).

Cytokine measurements

Cytokine levels were assessed on days 1 to 3 in culture supernatants, using cytometric Cytokine Bead Arrays (CBA; BD Biosciences). Samples were analyzed with a FACSCanto II system and FCAP Array 1.0.1 software from BD Biosciences.

For intracellular staining of IFN- γ , cells (1.5 \times 10⁶/mL) were stimulated in RPMI-10% FCS with IL-2 (100 U/mL) or mevastatin (5 μ mol/L) plus IL-2 for 2 to 3 days. During the last 3 to 4 hours, cells were treated with Brefeldin A (BFA; BD Biosciences) and were then harvested, washed, and stained with fluorochrome-conjugated anti-CD3 monoclonal antibody (mAb) or anti-CD56 mAb for 30 minutes at 4°C. Cells were washed twice and treated with FIX&PERM (An der Grub Bioresearch). Fixed, permeabilized cells were stained with fluorochrome-conjugated anti-IFN- γ antibody. After 2 more washes, the cells were analyzed with a FACSCanto II system.

Apoptosis assay

Culture supernatants were analyzed for the presence of caspase-cleaved cytokeratin-18 on days 2 to 3. Cytokeratin-18 is selectively expressed in cells of epithelial origin. A specific antibody was used to detect a neoepitope of cytokeratin-18 (CK18-Asp396), which is released after cleavage by multiple caspases during apoptosis (M30 CytoDeath ELISA, PEVIVA).

Statistical analyses

Group comparisons were carried out using Student's *t* test. All calculated *P* values are results of 1-sided tests. A value of *P* \leq 0.05 was considered statistically significant. Microsoft Excel was used for calculations.

Results

Statins and IL-2 synergistically induce IFN- γ production in CD56⁺ human PBMCs

In extension of our previous studies of DC-mediated $\gamma\delta$ T-cell activation (13), we used statins to inhibit the first committed step of the mevalonate pathway catalyzed by

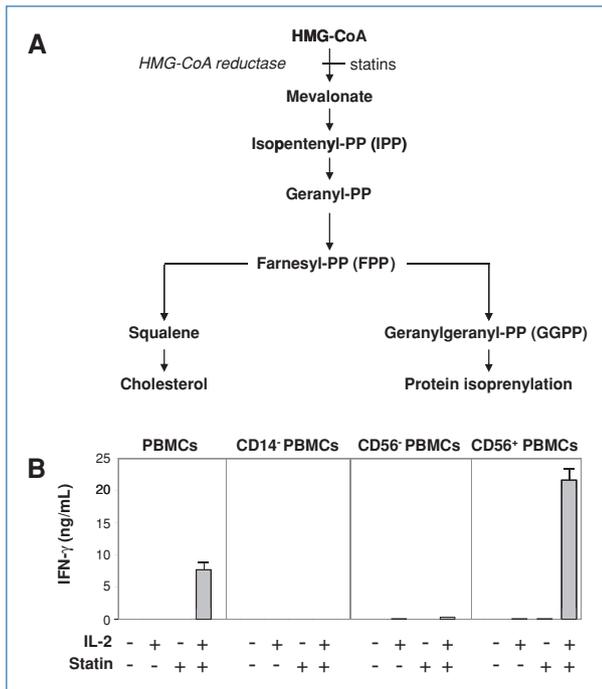


Figure 1. Synergistic induction of IFN- γ production in CD56⁺ PBMCs by statin and IL-2. A, mevalonate metabolic pathways. B, total PBMCs or subsets (1×10^6 /mL) were either left untreated or stimulated with IL-2 (100 U/mL), mevastatin (5 μ mol/L) or mevastatin plus IL-2. IFN- γ levels were determined in culture supernatants on day 2. One of 2 independent experiments with similar results is shown (results are mean \pm SD).

HMG-CoA reductase (Fig. 1A; refs. 3, 4). We surprisingly observed that mevastatin induced IFN- γ production in human PBMCs when IL-2 was present (Fig. 1B). The response was

concentrated within the CD56⁺ subset and was completely abrogated when CD14⁺ or CD56⁺ cells were depleted from PBMCs prior to stimulation (Fig. 1B).

In all subsequent experiments, we focused on the CD56⁺ subset of PBMCs to further study the role of mevalonate pathway intermediates in the interactions between CD56⁺ innate lymphocytes and CD56⁺ APCs. In 16 different donors, $11.7 \pm 2.5\%$ of total PBMCs were CD56⁺. CD56⁺ PBMCs consisted mainly of NK cells ($52.5 \pm 19.5\%$) and also of a CD3⁺ T-cell subset ($23.6 \pm 7.3\%$), which harbored a substantial $\gamma\delta$ T-cell population ranging from 20% to 69.5% of all CD56⁺ T cells (13). Moreover, we and others have shown that CD56⁺ PBMCs also harbor CD14⁺ monocyte/DC-like APCs ($10.7 \pm 5.7\%$; Fig. 2A; refs. 13–15).

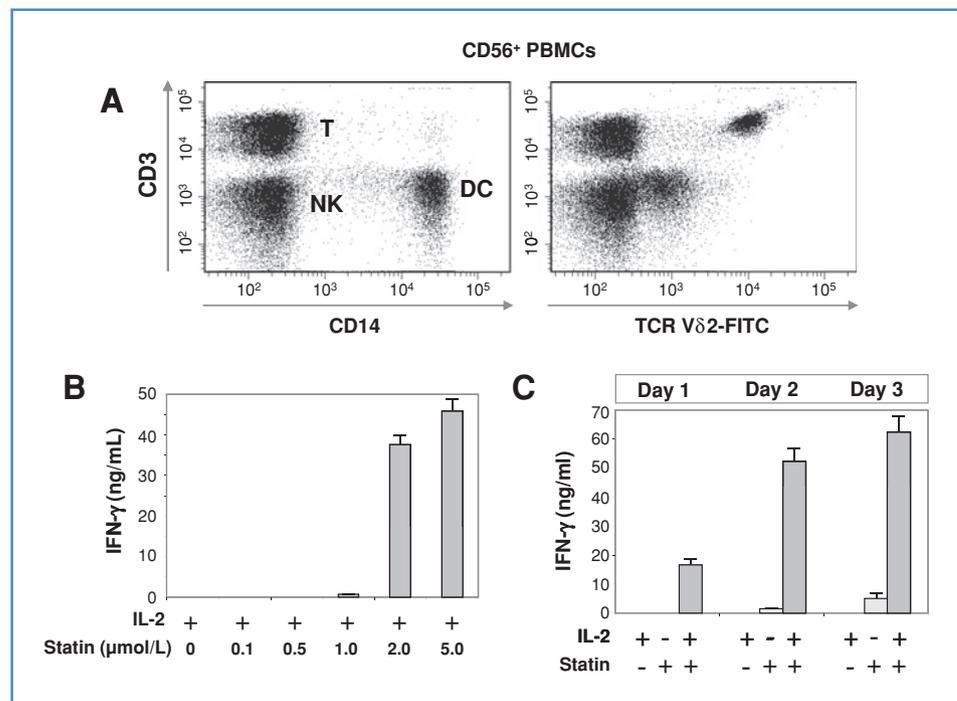
Dose and time dependence of statin-induced IFN- γ production

Experiments using increasing concentrations of statin revealed that the dose–response relationship was nonlinear and more likely reflected an on-off mechanism with a threshold between 1 and 2 μ mol/L of statin (Fig. 2B). The response was rapid and resulted in already substantial amounts of IFN- γ within 24 hours and in large amounts of IFN- γ within 2 to 3 days (Fig. 2C).

Specificity of the statin effect to HMG-CoA reductase inhibition and to suppression of protein prenylation

To ascertain whether the effect of statin was specific to the inhibition of HMG-CoA reductase, the cultures were supplemented with mevalonate, the immediate product of HMG-CoA reductase, which allows the biosynthetic pathway to proceed despite statin-mediated inhibition of HMG-CoA reductase. As shown in Figure 3A, mevalonate at 10 μ mol/L

Figure 2. Synergistic induction of IFN- γ production in CD56⁺ PBMCs by statin and IL-2: dose and time dependence. A, cellular subsets present within CD56⁺ PBMCs. CD56⁺ cells were isolated from total PBMCs, using CD56 microbeads. CD56⁺ PBMCs were then stained for CD3 and CD14 (left) or CD3 and TCR V δ 2 (right). B, statin-dose dependent IFN- γ production of CD56⁺ PBMCs was measured on day 2. C, time-dependent IFN- γ production of CD56⁺ PBMCs. Results were combined from 3 independent experiments (results are mean \pm SD).



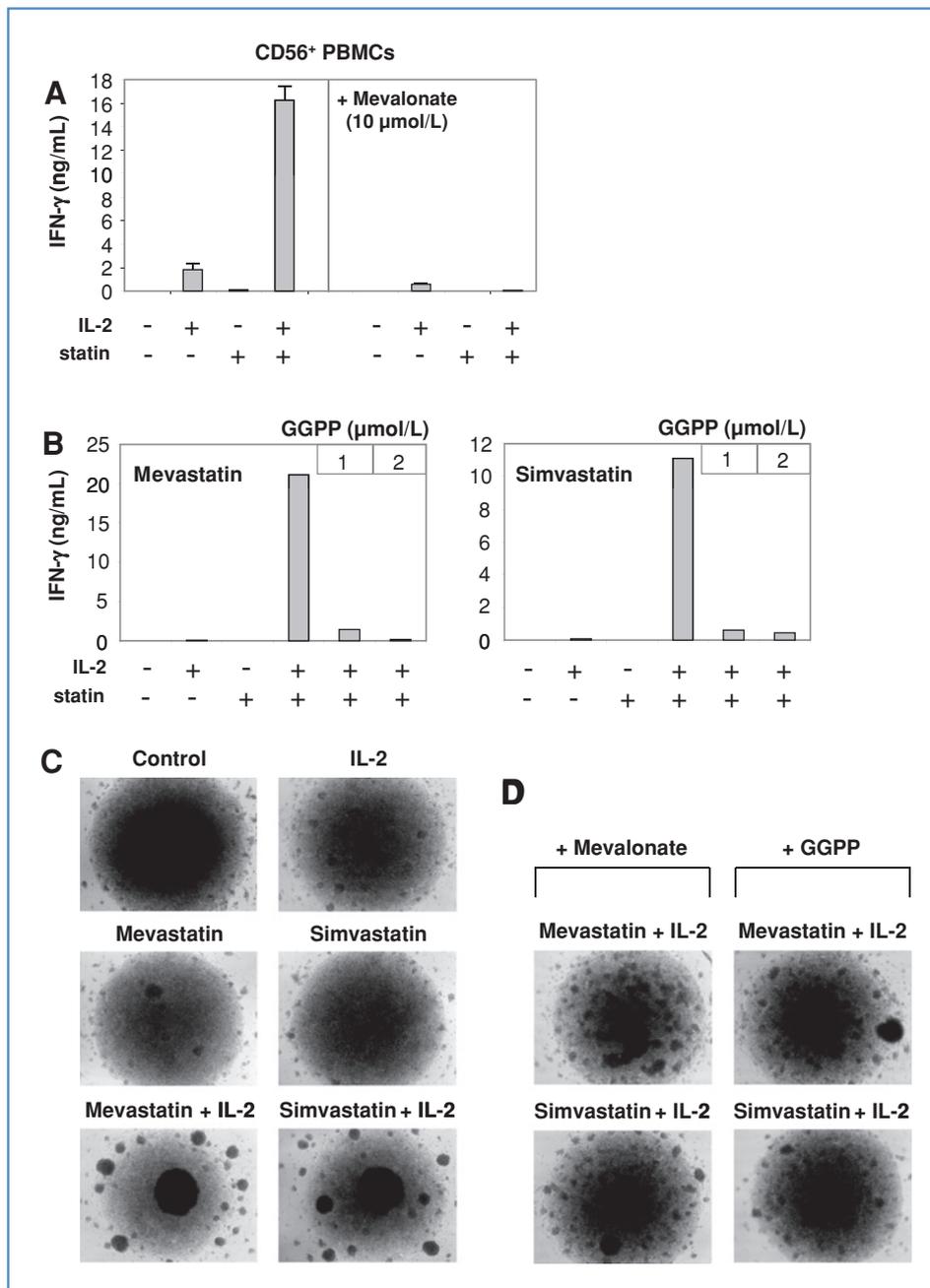


Figure 3. Abrogation of the IFN- γ response induced by statin plus IL-2 by mevalonate or GGPP supplementation. CD56⁺ PBMCs (1×10^6 /mL) were stimulated with IL-2 (100 U/mL), statin (5 μ mol/L), or statin plus IL-2. A, supplementation with mevalonate. B, supplementation with GGPP. C, microscopic examination of CD56⁺ PBMC aggregation in response to statin plus IL-2. D, abrogation of cell aggregation in the presence of mevalonate and GGPP. One of 3 independent experiments is shown.

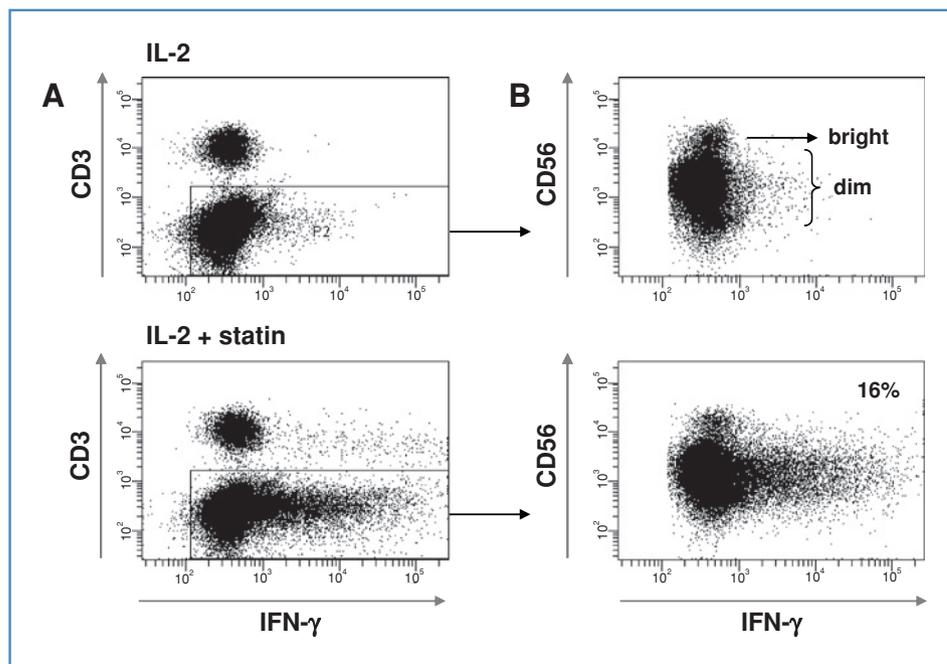
almost totally prevented the IFN- γ response induced by statin plus IL-2.

Statins inhibit the mevalonate pathway prior to divergence of the cholesterol biosynthesis and protein prenylation branch (Fig. 1A). To further investigate the specificity of statin-induced IFN- γ production and to distinguish the relative importance of the various branches of the mevalonate pathway, cultures of CD56⁺ PBMCs were treated with mevastatin plus IL-2 or simvastatin plus IL-2 (18) and concomitantly supplemented with prenyl pyrophosphates. As shown in Figure 3B, GGPP at 1 to 2 μ mol/L almost totally prevented

the IFN- γ response induced by statin plus IL-2 (>90% reduction; $P = 0.001$). In contrast, FPP had no effect at all (not shown). For additional control purposes, we also used YM-53601 to specifically inhibit squalene synthase (27), the rate-limiting enzyme of cholesterol biosynthesis (Fig. 1A). YM-53601 in combination with IL-2 completely failed to induce IFN- γ production (not shown), excluding the possibility that statin-mediated inhibition of the cholesterol biosynthesis branch also leads to NK cell activation.

Microscopic examination of cell cultures in round-bottom wells confirmed the synergistic stimulatory effects of statin

Figure 4. CD56^{dim} NK cells produce IFN- γ in response to stimulation with statin plus IL-2. A, CD56⁺ PBMCs (1.5×10^6 /mL) were stimulated for 48 hours with either IL-2 alone (100 U/mL) or with mevastatin (5 μ mol/L) plus IL-2. Cells were stained for intracellular IFN- γ and for surface CD3 or CD56 (B).



plus IL-2 on CD56⁺ PBMC aggregation (Fig. 3C) and confirmed abrogation of the cellular response by mevalonate or GGPP supplementation (Fig. 3D).

CD56^{dim} NK cells produce IFN- γ in response to statin plus IL-2

To identify the cellular source of IFN- γ , we performed intracellular staining of IFN- γ in CD56⁺ PBMCs after stimulation with statin plus IL-2. Cells were counterstained for CD3 and CD56 to distinguish T from NK cells as well as CD56^{bright} from CD56^{dim} NK cells (28, 29). Data shown in Figure 4A clearly indicate that IFN- γ was almost exclusively produced by CD3⁻ CD56⁺ cells (i.e., NK cells). Among NK cells, only CD56^{dim} cells (i.e., cytotoxic NK cells) produced IFN- γ (Fig. 4B).

CD56⁺ monocyte/DC-like cells are required for NK cell activation in response to statin plus IL-2

We then investigated the IFN- γ response as an indication of the interaction between NK cells and monocyte/DC-like cells known to be present within the subset of CD56⁺ PBMCs (13–15). Phenotypic analyses revealed that freshly isolated monocyte/DC-like cells expressed not only higher levels of CD14 than conventional CD56⁻ CD14⁺ peripheral blood monocytes but also significantly higher levels of MHC class I (HLA-ABC), MHC class II (HLA-DR), costimulatory (CD86), and adhesion (CD54) molecules (Supplementary Fig. S2). Moreover, these monocyte/DC-like cells substantially upregulated all surface markers within 1 day of culture in the absence of exogenous factors and presented with even higher levels of these molecules after statin treatment (Supplementary Fig. S2). Slan (M-DC8; 6-Sulfo LacNAc) is a carbohydrate modification of P-selectin glycoprotein ligand-1 (PSGL-1), which is expressed on CD14^{low} CD16⁺ monocyte/DC-like cells (30, 31). Although Slan was readily detectable on 3% to 4% of CD56⁻ peripheral blood

monocytes, we failed to detect Slan on CD56⁺ monocyte/DC-like cells. Likewise, CD56⁺ monocyte/DC-like cells also lacked CD1c (BDCA-1), which is expressed on a major subpopulation of human myeloid blood DCs (data not shown; ref. 32).

To examine the functional role of CD56⁺ monocyte/DC-like cells, we tested the response of CD56⁺ PBMCs that had been depleted of CD14⁺ cells prior to stimulation with statin plus IL-2. Importantly, depletion of CD56⁺ CD14⁺ monocyte/DC-like cells was accompanied by a complete loss of IFN- γ (Fig. 5A), IL-1 β , and IL-6 production (not shown).

To test whether CD56⁺ monocyte/DC-like cells are sufficient for NK cell activation in response to statin plus IL-2, CD56⁺ PBMCs were isolated and CD56⁺ DC-like cells (CD14⁺ CD3⁻), NK cells (CD3⁺ CD14⁻), and T cells (CD3⁺ CD14⁻) were sorted with a FACS system. The individual cell populations were remixed and cocultured in the presence of statin plus IL-2. The results shown in Supplementary Figure S3 confirm that CD56⁺ DC-like cells are both required and sufficient to activate NK cells. However, in the presence of T cells, the response was further enhanced, indicating that T cells can also contribute to the observed response.

When sorted CD56⁺ DC-like cells and NK cells were seeded separately in the upper or lower chamber of transwells, respectively, to prevent cell-to-cell contact, the levels of IFN- γ were significantly lower (≤ 20 ng/mL) than the levels of IFN- γ present in remixed cultures of CD56⁺ DCs and NK cells (60 ng/mL). However, IFN- γ production was still substantial under conditions of physical separation, indicating that cell-to-cell contact is not required but can enhance IFN- γ production.

To clarify whether CD56⁺ CD14⁺ monocyte/DC-like cells are especially adept at activating NK cells in response to statin plus IL-2, we compared various APCs as accessory cells. For this purpose, CD56⁺ PBMCs were depleted of CD14⁺ cells to

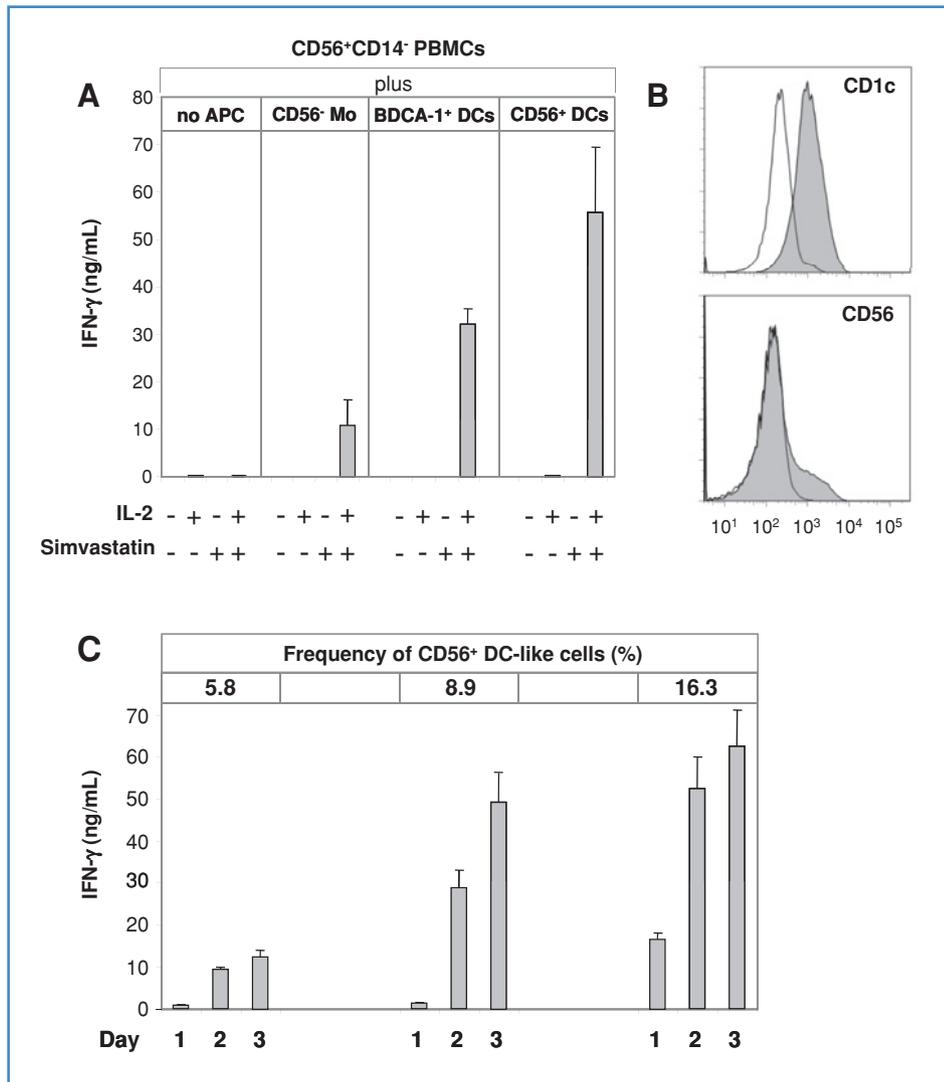


Figure 5. IFN- γ production induced by statin plus IL-2 depends on CD56⁺ monocyte/DC-like cells. A, CD56⁺ PBMCs depleted of CD14⁺ cells (1×10^6 /mL) were reconstituted with various APCs (1.5×10^4 /mL) and were stimulated with IL-2 (100 U/mL), statin (5 μ mol/L), or statin plus IL-2. B, CD1c and CD56 expression by BDCA-1⁺ DCs (filled histograms; isotype controls: open histograms). C, IFN- γ levels depending on the frequency of monocyte/DC-like cells in individual CD56⁺ PBMC preparations. Results shown in A and C each derive from 3 independent experiments (results are mean values \pm SD).

eliminate monocyte/DC-like cells and were then reconstituted with consistent numbers of BDCA-1⁺ (CD1c⁺) myeloid DCs, CD14⁺ monocytes that had been depleted of CD56⁺ cells (i.e., CD56⁻ monocytes), or CD56⁺ monocyte/DC-like cells. As shown in Figure 5A, BDCA-1⁺ myeloid DCs also served as accessory cells, although they were somewhat less potent than CD56⁺ monocyte/DC-like cells. Control stainings shown in Figure 5B surprisingly revealed partial expression of CD56 by BDCA-1 myeloid DCs. Conventional blood monocytes, which lack CD56, exhibited the lowest accessory cell capacity (Fig. 5A).

The importance of CD56⁺ monocyte/DC-like cells was also reflected by the correlation between the frequency of monocyte/DC-like cells within the CD56⁺ PBMCs and the actual level of IFN- γ production (Fig. 5B). IFN- γ production in CD56⁺ cell preparations from donors containing a rather small monocyte/DC population (\sim 5%) was clearly less efficient than IFN- γ production in CD56⁺ cell preparations from donors containing a relatively large monocyte/DC population (\sim 15%; Fig. 5B).

IFN- γ production in response to statin plus IL-2 depends on endogenous IL-18, IL-1 β , and caspase-1

IL-12 is a major IFN- γ -inducing cytokine. In our cytokine analyses, we failed to detect IL-12 in supernatants of cultures stimulated with statin plus IL-2. In addition, IL-12p70-neutralizing antibodies completely failed to inhibit IFN- γ production, indicating that IL-12 is not involved (not shown). In contrast, antibodies neutralizing IL-18 bioactivity strongly reduced IFN- γ production induced by statin plus IL-2 in cultures of CD56⁺ PBMCs ($P = 0.002$; Fig. 6A). Although somewhat less effective, neutralization of IL-1 β also inhibited IFN- γ production ($P = 0.009$). Moreover, concomitant neutralization of both IL-18 and IL-1 β abolished IFN- γ production almost completely ($P = 0.002$; Fig. 6A). Because both cytokines require caspase-1 for activation (33, 34), we also tested the effects of the caspase-1 inhibitor YVAD (35) on IFN- γ production induced by statin plus IL-2. YVAD alone was capable of completely abrogating the IFN- γ response ($P = 0.002$; Fig. 6A). Both mevastatin and simvastatin acted in synergy with IL-2 to

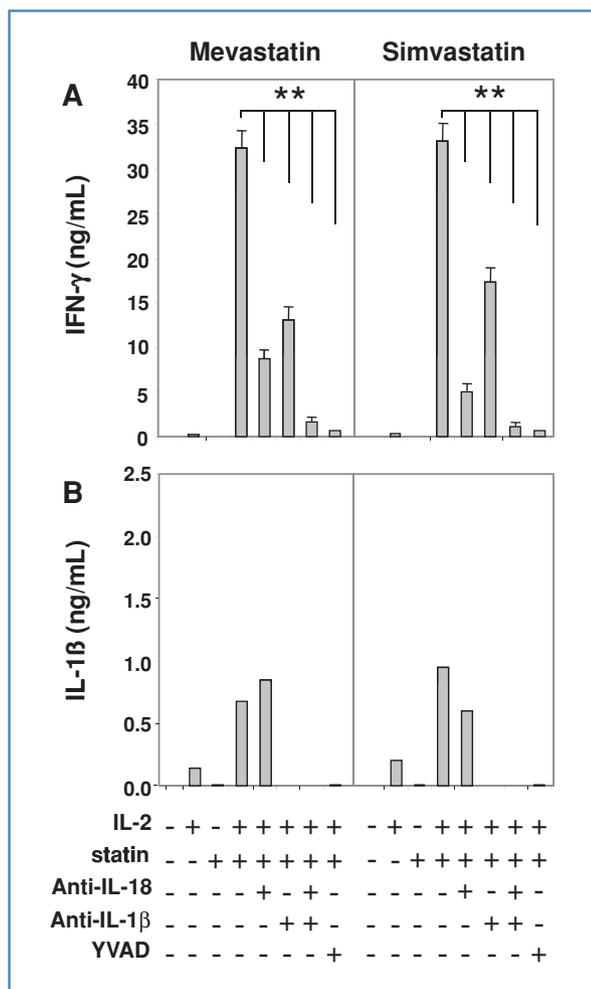


Figure 6. The IFN- γ response induced by statins in combination with IL-2 depends on endogenous IL-18, IL-1 β , and caspase-1. A, CD56⁺ PBMCs (1×10^6 /mL) were treated as indicated and IFN- γ was determined in culture supernatants on day 3. Three independent experiments, asterisks indicate statistical significance between the control groups and the groups that received neutralizing/blocking reagent according to Student's *t* test (**, $P < 0.01$). B, IL-1 β was determined in pooled supernatants from triplicate cultures. One of 2 experiments with similar results is shown.

induce the production of IL-1 β (Fig. 6B). Collectively, the data indicated that IL-18 and IL-1 β are the endogenous mediators of the statin-induced IFN- γ response.

CD56^{dim} NK cells activated in the presence of statin have limited degranulation capacity

In addition to the cytokine-producing capacity, we wanted to assess the cytotoxic activity of NK cells in our system. Previous work has demonstrated that statins may inhibit NK cell degranulation and cytotoxicity (10). One way to assess the cytotoxic function of NK cells is to measure the mobilization of CD107a (lysosome-associated membrane protein-1, LAMP-1) to the cell surface by flow cytometry (Supplementary Methods; 36). Consistent with previous reports (10), we found that

cytokine-induced CD107a surface exposure was prevented in statin-treated NK cells (Supplementary Fig. S4).

NK cell-derived IFN- γ and statin cooperate to generate potent cytotoxic effects

In addition to the degranulation capacity, we wanted to study the cytotoxic antitumor effects of NK cells activated with statin plus IL-2. For this purpose, we used A-498 cells, a well-characterized example of human RCC (19–21, 37), and cultured them in 96-well round-bottom plates. CD56⁺ PBMCs were then added and stimulated with statin and IL-2. While the A-498 cell monolayer seemed relatively intact in the presence of statin or IL-2 alone, the combination of statin and IL-2 induced an almost complete destruction of the epithelial cell monolayer indicative of potent cytotoxicity (Fig. 7A). However, the observed strong cytotoxicity of CD56⁺ PBMCs stimulated with statin plus IL-2 was in apparent contrast with the inhibitory effects of statin on NK cell degranulation (Supplementary Fig. S4; ref. 10) and suggested that other mechanisms were active.

To examine the role of soluble factors generated during stimulation of CD56⁺ PBMCs in the cytotoxicity against A-498 cells, the effects of cell-free conditioned media (CM) on A-498 cells were examined. The ability of soluble factors contained in CM to induce apoptosis of A-498 cells was investigated by measuring the release of caspase-cleaved cytokeratin-18 (CK18-Asp396) from apoptotic carcinoma cells (Fig. 7B). Spontaneous apoptosis of A-498 cells in the absence of CM resulted in low levels of CK18-Asp396. Addition of CM from CD56⁺ PBMC control cultures (CM-Co) enhanced A-498 cell apoptosis ($P = 0.008$; Fig. 7B). Moreover, CM from CD56⁺ PBMCs stimulated with IL-2 (CM-IL-2) substantially increased A-498 cell apoptosis compared with CM-Co ($P = 0.03$). CM from CD56⁺ PBMCs treated with mevastatin (CM-mev) also enhanced tumor cell apoptosis compared with CM-Co ($P = 0.01$), suggesting that mevastatin directly induced tumor cell apoptosis. However, compared with CM-IL-2 or CM-mev, it significantly enhanced A-498 cell apoptosis when CM from CD56⁺ PBMCs stimulated with statin plus IL-2, which contains high levels of IFN- γ , was used ($P = 0.0008$ and $P = 0.004$, respectively; Fig. 7B), indicating that soluble factors present in CM cooperate to exhibit potent cytotoxic effects directed against human renal carcinoma cells.

We therefore tested the direct effects of statin and recombinant IFN- γ on human A-498 cells. Statin alone was capable of inducing A-498 cell apoptosis ($P = 0.002$), confirming that statin present in CM directly acts on tumor cells (Fig. 7B). IFN- γ alone had little effect on tumor cell apoptosis (not shown). However, the combination of statin and IFN- γ resulted in strong cytotoxicity ($P = 0.009$; Fig. 7B).

To further establish the role of IFN- γ in statin-induced cytotoxicity, we used specific antibodies to neutralize natural IFN- γ and recombinant IFN- γ . While CM from CD56⁺ PBMCs stimulated with statin plus IL-2 caused serious damage to the A-498 tumor cells, neutralization of IFN- γ contained in these CM resulted in the almost complete restoration of the tumor cell monolayer (Fig. 7C, left), confirming the important role of IFN- γ in statin-mediated cytotoxicity. Likewise, statin and

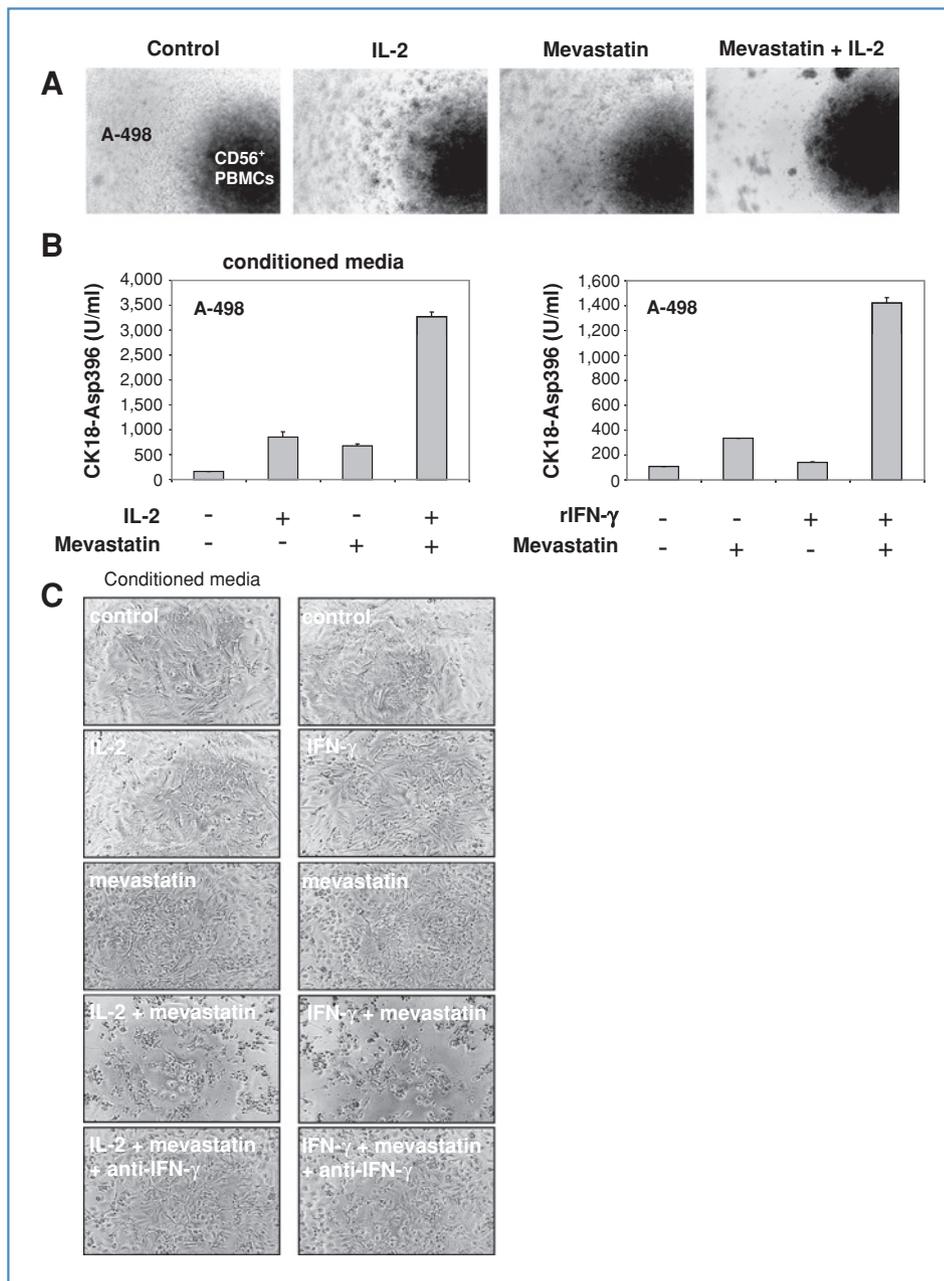


Figure 7. NK cell-derived IFN- γ and statin cooperate to generate potent cytotoxic effects. A, CD56⁺ PBMCs (1×10^6 /mL) were cocultured with A-498 human RCC cells and were stimulated with IL-2 (100 U/mL), mevastatin (5 μ mol/L), or mevastatin plus IL-2. B, cell-free CM from CD56⁺ PBMCs was added to A-498 cultures and apoptosis of epithelial cells was measured. A-498 cultures were also treated with mevastatin (5 μ mol/L), recombinant IFN- γ (100 U/mL), or statin plus IFN- γ and apoptosis was determined (mean \pm SD). C, microscopic examination of A-498 cells treated either with CM (left) or with statin \pm recombinant IFN- γ (right). One of 3 independent experiments with similar results is shown.

recombinant IFN- γ cooperated to cause substantial damage to A-498 tumor cells and neutralization of recombinant IFN- γ under these conditions almost totally prevented cytotoxicity (Fig. 7C, right).

To extend the relevance of the observed cytotoxic effects, we used additional cancer cell lines. In MCF-7 cells, which are a well-studied example of breast cancer, statin-induced growth inhibition and cytotoxic effects have previously been demonstrated (22, 23). Data shown in Supplementary Fig. S5 show that medium conditioned by CD56⁺ PBMCs treated with statin plus IL-2 caused severe damage to the MCF-7 cells (Supplementary Fig. S5A) and induced high levels of CK18-Asp396, indicative of MCF-7 cell apoptosis (Supplementary

Fig. S5B, left). Moreover, statin and rIFN- γ cooperated to induce potent direct cytotoxic effects on MCF-7 cells (Supplementary Fig. S5B, right). The cooperative cytotoxic effect of statin and rIFN- γ was also observed in the well-characterized prostate cancer cell line DU-145 (24) but not in LNCaP prostate cancer cells (Supplementary Fig. S5C), which have previously been shown to be IFN- γ nonresponsive (25) due to a lack of *JAK1* gene expression (26).

Discussion

We show, for what we believe to be the first time, potent stimulatory effects of statins on human NK cells.

Statin-mediated NK cell activation depended on IL-2 costimulation and on the presence of CD56⁺CD14⁺ monocyte/DC-like accessory cells (Supplementary Fig. S6). The observed stimulatory effects were specific to the inhibition of protein prenylation, as NK cell activation was completely reversible by supplementation with GGPP (Fig. 3), the substrate of geranylgeranyl transferase, which catalyzes the covalent attachment of prenyl groups to proteins such as the Rho GTPases of the RAS superfamily (2).

Our experiments with neutralizing antibodies revealed that the IFN- γ response induced by statin plus IL-2 was mediated by endogenous IL-18 and IL-1 β (Fig. 6), which are known to be produced by myeloid APCs but not by NK cells, also indicating that statins primarily acted on APCs. IL-18 alone is a poor inducer of IFN- γ production by NK cells (29); however, IL-18 synergizes with IL-2 to induce IFN- γ production by NK cells *in vitro* (38) and *in vivo* (39). IL-18 can induce IFN- γ mRNA, but secretion of its protein product requires costimulation with IL-2 (40).

The effects of statins on NK cells reported so far were all inhibitory (9–12). In all these studies, which focused on NK cell–target cell interactions, purified NK cells were used in coculture with target cells but the role of APCs had not been investigated at all. We show here that in the presence of distinct APCs of the monocyte/DC lineage, which share CD56 expression with NK cells, statins induce IFN- γ production in IL-2–activated NK cells, resulting in considerable cytotoxicity (Fig. 7). We show that statins directly acted on human carcinoma cells to induce apoptosis and that IFN- γ produced by NK cells cooperates with statin to enhance tumor cell death (Fig. 7). These data clearly indicated that statin and NK cell–derived IFN- γ can cooperate to exert potent antitumor activity (Fig. 7).

In the majority of animal tumor models, IFN- γ was indispensable for tumor rejection (41, 42). In several of these studies, neither perforin nor Fas ligand was found to be essential for tumor rejection. In one study, adoptively transferred CD8⁺ effector T cells could reject tumors in an IFN- γ –dependent fashion despite multiple cytolytic defects (43). In a recent study, chemotherapy was shown to make tumor cells more susceptible to the cytotoxic effects of CTLs (44). Chemotherapy induced a perforin-independent dramatic increase in permeability to CTL-derived granzyme B also resulting in a spreading of cytotoxicity to neighboring tumor cells. Similarly, our present data demonstrate that statins can sensitize tumor cells to cytotoxic effects of NK cell–derived IFN- γ (Fig. 7), thus generating a potent antitumor mechanism that is perforin independent and does not even require cell-to-cell contact between effector cells and tumor cells.

We and others have previously described CD56⁺ monocyte/DC-like cells (13–15), which share properties of monocytes and

of DCs (Supplementary Fig. S2). The ability of CD56⁺ monocyte/DC-like cells both to induce an allogeneic MLR (14) and to activate $\gamma\delta$ T cells (13) as well as NK cells (Fig. 4) suggested that these APCs may already have DC-like properties and may further differentiate toward mature DCs during culture (Supplementary Fig. S2). Recently, CD56 expression has also been detected on monocyte-derived DCs generated in the presence of type 1 IFN and granulocyte macrophage–colony stimulating factor (45), suggesting that the CD56⁺CD14⁺ monocyte/DC-like cells described here represent the *in vivo* counterpart of the CD56⁺ monocyte–derived IFN DCs (45).

In side-by-side analyses, we found that BDCA-1⁺ (CD1c⁺) myeloid DCs (32) could also serve as accessory cells in statin-induced NK cell activation, although they were slightly less potent than the CD56⁺CD14⁺ monocyte/DC-like cells (Fig. 5A). Control stainings of BDCA-1⁺ DCs unexpectedly revealed that they partially expressed CD56 (Fig. 5B), which may explain their capacity to mediate NK cell activation. Among CD14⁺ cells, only CD56⁺ monocyte/DC-like cells could effectively mediate statin-induced NK cell activation whereas conventional peripheral blood monocytes, which lack CD56, were rather ineffective (Fig. 5A).

Collectively, these observations suggest that CD56⁺ monocyte/DC-like cells preferentially mediate NK cell activation in response to statins. Together with our recent work on $\gamma\delta$ T cells (13), the present data also suggest that CD56⁺ DC-like cells may be particularly effective at inducing innate lymphocyte activation, a property that may be harnessed for cancer immunotherapy.

Disclosure of Potential Conflict of Interest

The authors have declared that no conflict of interest exists.

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