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Dopamine Inhibits the Effector Functions of Activated NK Cells via the Upregulation of the D5 Receptor

Joanna Mikulak,*1 Luisa Bozzo,*1 Alessandra Roberto,* Elena Pontarini,* Paolo Tentorio,* Kelly Hudspeth,* Enrico Lugli,* and Domenico Mavilio*1†

Several lines of evidence indicate that dopamine (DA) plays a key role in the cross-talk between the nervous and immune systems. In this study, we disclose a novel immune-regulatory role for DA: inhibition of effector functions of activated NK lymphocytes via the selective upregulation of the D5 dopaminergic receptor in response to prolonged cell stimulation with rIL-2. Indeed, engagement of this D1-like inhibitory receptor following binding with DA suppresses NK cell proliferation and synthesis of IFN-γ. The inhibition of IFN-γ production occurs through blocking the repressor activity of the p50/c-REL dimer of the NF-κB complex. Indeed, the stimulation of the D5 receptor on rIL-2–activated NK cells inhibits the binding of p50 to the microRNA 29a promoter, thus inducing a de novo synthesis of this miRNA. In turn, the increased levels of microRNA 29a were inversely correlated with the ability of NK cells to produce IFN-γ. Taken together, our findings demonstrated that DA switches off activated NK cells, thus representing a checkpoint exerted by the nervous system to control the reactivity of these innate immune effectors in response to activation stimuli and to avoid the establishment of chronic and pathologic inflammatory processes. The Journal of Immunology, 2014, 193: 2792–2800.

The central and peripheral nervous systems are able to modulate immune responses by releasing several neurotransmitters, neuropeptides, hormones, and cytokines (1, 2). Dopamine (DA) emerged as one of the catecholamines playing a central role in the neuremodulation of the immune system. Indeed, both primary and secondary lymphoid organs contain high levels of dopaminergic nerve terminals (3–5). Moreover, many circulating immune cells, including monocytes, neutrophils, eosinophils, B cells, T cells, NK cells, and dendritic cells (DCs), constitutively expresses DA receptors (DARs) on their surface (6–10). The engagement of DARs on these immune cell compartments is associated with the regulation of several important cell functions, such as cellular proliferation and division, cytokine production, cell polarization, and migration (11–17).

Five DARs have been identified and classified into two subfamilies on the basis of their pharmacological profiles and second messengers involved in their downstream signaling (18–20). D1 and D5 DARs belong to the so-called D1-like subfamily coupled with the stimulatory α subunit of G protein, whose triggering is associated with the production of cAMP. The D2-like subfamily includes D2, D3, and D4 DARs that are coupled with inhibitory Gαs, suppressing the synthesis of cAMP. However, D1- and D2-like DARs also can be coupled with different Gα subunits associated with other downstream pathways, thus explaining the existence of heterogeneous or even opposite functional outcomes in the context of immune cell populations expressing similar phenotypic distributions of DARs (9, 21–24).

NK cells eliminate non-self targets, such as virus-infected or tumor-transformed cells, and produce proinflammatory mediators like IFN-γ in the absence of a prior sensitization to specific Ags. These functions are controlled by a complex process based on the dynamic balance between activating and inhibitory signals that are delivered simultaneously by several NKRs (25–27). Impairment of these synergic interactions lead to disease progression and aberrant immune responses (26). DARs are constitutively expressed on both human and murine NK cells where they modulate cytotoxicity, as observed in rats with a hyperreactive dopaminergic system and impaired NK cell functional responses (8, 28–30). Despite this experimental evidence, very little is known about the phenotypic distribution of DARs or the effects of DA on the homeostasis and effector functions of human resting and activated NK cells.

The present study fully characterizes the repertoire and the functional correlates of DARs expressed on resting and activated NK cells following binding with DA. We demonstrate that the activation of the dopaminergic pathway suppresses NK cell proliferation and secretion of IFN-γ. This phenomenon occurs only in activated NK cells through the selective engagement of D5 receptor, which is significantly upregulated in response to stimulation with rIL-2. We also demonstrate that the D5-mediated inhibition of IFN-γ is associated with an increased level of microRNA 29a (miR-29a), which was recently reported to be a suppressor of IFN-γ production in human NK cells (31).
Materials and Methods

Isolation and activation of NK cells

Human PBMCs were obtained from buffy coats of healthy volunteers who signed consent forms in accordance with the Declaration of Helsinki and with clinical protocols approved by the Institutional Review Board of Desio Hospital, Milan, Italy. PBMCs were isolated over Ficoll-Paque Premium density gradients (GE Healthcare Bio-Sciences). CD56+ NK cells were isolated by a negative cell-sorting technique (Stem Cell Tech) using CD56 microbeads (Miltenyi). Cells were washed and re-suspended in 0.1% sodium citrate to prevent clumping. The viability of NK cells was assessed by Annexin V staining (BD Biosciences) and was above 95%. CD56+ NK cells were activated using 10 ng/mL PMA, 2μg/mL ionomycin and 2 μM 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (Invitrogen) and cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin/streptomycin, and with 200 IU/ml human rIL-2 (Roche Molecular Biochemicals) for activation (35).

Cell lines

Human erythroleukemia K562 and HEK-293 cell lines were purchased from the American Type Culture Collection and cultured in DMEM with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin/streptomycin, and 10 mM HEPES. EndoGRO HUVECs (Millipore) were cultured in EndoGRO medium. The NT2 human teratocarcinoma cell line, kindly provided by Prof. Jacopo Meldolesi (San Raffaele Institute, Milan, Italy), was cultured in DMEM medium with 10% FetalClone III serum, 2 mM l-α-ketoglutarate, and 100 U/ml penicillin/streptomycin.

Dopaminergic agonist and antagonist

Dopamine hydrochloride (Sigma-Aldrich) was used at a concentration between 10⁻⁷ and 10⁻¹⁸ M. The following dopaminergic DAR agonists were used: SKF 38393 (0.1 μM) to trigger D1/D5 receptors, (-)-quinpirole hydrochloride (20 μM) to trigger D2 receptor, 7-hydroxy-DPAT hydrobromide (10 μM) to trigger D3 receptor, and PD 168077 maleate (20 μM) to trigger D4 receptor (Tocris Bioscience and R&D Systems). The following DAR antagonists were used: SCH 23390 anti-D1/D5 receptors (1, 0.1, or 0.001 μM), L-741,626 anti-D2 receptor (1 μM), U99194A anti-D3 receptor (0.1 μM), and L-741,742 anti-D4 (3 μM); all from Sigma-Aldrich. The optimal doses of DAR blockers and antagonists were determined by testing their toxicity through flow cytometry experiments assessing NK cell death and apoptosis at different concentrations (data not shown).

Flow cytometry

For multicolor flow cytometric analysis, NK cells were stained with the following conjugated mAbs: FITC-labeled CD3, PC5-labeled CD56, PE-labeled NKp46, NKp30, NKp44, NKGD2, NK2A, CD94, NKp80, 2B4, KIR2DL2/D3L/D2S, KIR2DL1/D1S, KIR3DL1/D1S, Siglec-7, and LIR1/ILT2 (all from Beckman Coulter); PE-labeled NKG2C, NTB-A, and DNAM-1 (all from R&D Systems); PE-labeled LAIR-1 (BD Pharmingen); allotypocyanocyanin-Cy7-labeled CD16 and PE/Cy7-labeled CD14 (BD Biosciences Pharmingen); and allotypocyanocyanin-labeled CD16 (Miltenyi Biotec). For DAR staining, NK cells were incubated with 10 μg/ml total human IgG (Sigma-Aldrich) to avoid nonspecific binding, washed, and stained with appropriate unconjugated anti-D1, anti-D3, anti-D4, and anti-D5 (all from Millipore) or anti-D2 (Lifespan Biosciences) polyclonal Abs. Cells were washed and stained with an Alexa Fluor 488 goat anti-rabbit conjugated secondary Ab (Molecular Probes). An appropriate isotypic Ab labeled with Alexa Fluor was used as negative control. Live/Dead Aqua fluorescent reactive dyes (Invitrogen) were used to distinguish cell viability. A CD107a degranulation assay (34) was used to measure NK cell cytotoxicity.

To determine the cellular toxicity of the different doses of DA, we measured apoptotic or death rIL-2–activated NK cells using detecting Annexin V (FITC-labeled and/or propidium iodide (PI) assays (BD Pharmingen). Flow cytometric experiments were performed using a FACSCanto II (BD Biosciences), and the related data were analyzed using FlowJo software (TreeStar).

Cellular proliferation and secretion of IFN-γ

Proliferation of NK cells was measured by [3H]thymidine (GE Healthcare) uptake (16 h) in triplicate in the presence or absence of DA at different concentrations. For IFN-γ production, NK cells were incubated for 6 h, with or without K562 target cells at a 5:1 ratio, in the presence or absence of DA at different concentrations. Cytokine supernatants were harvested, filtered using Costar Spin-X centrifuge tube 0.22-μm filters (Corning BV), and IFN-γ released by NK lymphocytes was measured in cell supernatants by ELISA (R&D Systems).

Quantitative PCR analysis

Total RNA was extracted using RNeasy mini columns (QIAGEN, Valencia, CA), following the manufacturer’s instructions. One microgram of total RNA was used to generate cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR (qPCR) was done by the Fast SYBR Green method, using 10 ng template cDNA for the following primers: IFN-γ forward 5’-CTCTTG-GCTTTAGTCGCAAGG-3’ and IFN-γ reverse 5’-CTCCACACTTCTTTTG-GATGCT-3’ and S-18 forward 5’-AATCTTTAGGATTGATGCGCCTG-3’ and S-18 reverse 5’-CCTTGAGTTGTGACCCGGTTT-3’. All human DAR primers were purchased from OriGene Technologies. The expression of miR-29a and control U6 was detected using TaqMan MicroRNA specific assays (Applied Biosystems). Each sample was analyzed in triplicate and analyzed using an ABI 7900HT Sequence Detection System (Applied Biosystems).

Western blot analysis

Total cell protein extract in RIPA buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitor mixture was rocked for 30 min and then centrifuged (13,000 × g, 4 C, 15 min). Fixed amounts of protein, as determined by a Bradford assay or a Bradford assay kit (Bio-Rad, Hercules, CA), were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in 5% milk, incubated overnight at 4 C with the specific Abs, washed, and incubated with secondary goat anti-rabbit Ab conjugated with HRP. Western blot analysis was conducted according to standard procedures using an Immun-Star Western Chemiluminescence detection substrate kit (Bio-Rad, Hercules, CA). All rabbit polyclonal anti-DAR Abs were purchased from Millipore, and anti–β-actin Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Chromatin immunoprecipitation experiments

Chromatin immunoprecipitation (ChIP) was performed, according to the manufacturer’s protocol, using the EZ-Magna CHIP A/G kit (Millipore). Briefly, protein–DNA complexes in NK cells, treated or not with DA, were cross-linked with 1% formaldehyde, followed by glycine (0.125 M) treatment, and cells were harvested and resuspended in lysis buffer. Samples were sheared to 200–400-bp fragments by sonication using a Bioruptor Plus UCD-300 on high power for 35 cycles (30 s on/30 s off), and 10 μl each sample was taken as input. Then samples were treated with protein A beads and anti-p50 ChIP-grade Ab (Abcam); after purification, p50-associated DNA fragments were amplified by qPCR using the SYBR Green method and the following primers: miR-29a forward 5’-AGAGAATGAGAATGCCCGAAATTG-3’ and miR-29a reverse 5’-CAAATGATCCACAAAGCCTTGA-3’.

ChIP samples were analyzed in triplicate by qPCR and presented as the ratio between miR-29a gene quantification of NK cell lysate. Lysates were immune-precipitated with anti-p50 Ab either stimulated or not with DA and normalized with GAPDH of input.

Statistical analysis

All experiments were performed at least three times and analyzed in triplicate. The values of n independent experiments are specified in the figure legends. The statistical significance was assessed by the two-tailed unpaired Student t test using GraphPad software. Data are expressed as median ± SD.

Results

Distribution of DARs on freshly purified NK cells

The previously reported phenotypic distribution of DARs on freshly purified NK cells was assessed by gating CD94+ NK cells within total PBMCs using a flow cytometric approach (8). In addition to circulating NK cells, CD94 is constitutively expressed within total PBMCs using a flow cytometric approach (8). In addition to circulating NK cells, CD94 is constitutively expressed within total PBMCs using a flow cytometric approach (8). In addition to circulating NK cells, CD94 is constitutively expressed within total PBMCs using a flow cytometric approach (8). In addition to circulating NK cells, CD94 is constitutively expressed within total PBMCs using a flow cytometric approach (8). In addition to circulating NK cells, CD94 is constitutively expressed within total PBMCs using a flow cytometric approach (8).
resting NK cells. Nevertheless, our analyses revealed a different distribution of DARs; we found low levels of D5, D3, and D2 receptors and a high level of D4 receptor. Our experimental approach also revealed that the constitutive expression of DARs is restricted to CD56<sup>dim</sup>/CD16<sup>+</sup> NK cell subsets, because CD56<sup>bright</sup> cells were either negative or showed very low levels of these receptors (Fig. 1A, 1B).

The heterogeneous distribution of DARs on NK cells among different donors is likely associated with the low sensitivity of the currently commercially available anti-DAR polyclonal, indicating that flow cytometry is not the most reliable method to assess the quantitative expression of these receptors on the cell surface. Therefore, we confirmed the absence of the D1 receptor and the constitutive expression of the other four DARs, with D4 showing the highest amounts (Fig. 1D). These results are in line with a recent report showing similar levels of DAR transcripts also in splenic NK cells from mice that differ from their human counterparts only for the D1 receptor, whose mRNA was detected exclusively in murine NK cells (30).

DA inhibits NK cell effector functions via the upregulation of the D5 receptor following rIL-2 stimulation

Dopaminergic pathways are able to either modulate the homeostasis or induce the effector functions of several immune cell populations constitutively expressing DARs (16, 17). To validate this thesis on NK cells, freshly purified cells were cultured overnight with low doses of rIL-2 (20 IU) and then incubated with different concentrations of DA (10<sup>-2</sup>–10<sup>-18</sup> M) for 18 h. We then used flow cytometry to analyze the NK cell surface levels of a large panel of activating and inhibitory NKR, including natural cytotoxicity receptors, C-lectin type molecules, and killer Ig-like receptors. We did not observe any regulation of expression of the NKR repertoire in response to the incubation with DA. Furthermore, we also found that DA does not have any impact on NK cell proliferation, IFN-γ secretion/production (data not shown), or CD107a degranulation (data not shown). These results obtained with freshly purified NK cells are in contrast to those reported for resting murine NK cells, human DCs, and T lymphocytes, for which the engagement of DARs is associated with modulation of cell homeostasis and functional outcomes (16, 30).

On the basis of the previously reported DA-mediated inhibition of T cell proliferation following stimulation with anti-CD3 mAb
and rIL-2 (12, 14, 23, 37), we evaluated whether DA has a similar effect on activated NK cells. To this end, we stimulated NK cells with a high dose (200 IU) of rIL-2 for 5 d and measured the amount of \([\text{H}]\)thymidine uptake after 16 h in the presence or absence of different concentrations \((10^{-9} - 10^{-18} \text{ M})\) of DA. Treatment of rIL-2–activated NK cells with DA at \(10^{-9}, 10^{-12}, \text{ or } 10^{-15} \text{ M}\) induced a statistically significant inhibition of \([\text{H}]\)thymidine uptake. This phenomenon appeared to be dose dependent, with the highest level of inhibition observed at \(10^{-9} \text{ M}\) (Fig. 2A). To confirm that the decreased degree of NK cell proliferation was due to an effective immune-regulatory role for DA and was not associated with drug toxicity, we analyzed the frequencies of early apoptotic (Annexin V+/PI−) and dead (Annexin V+/PI+) NK cells using the same protocol for the above-mentioned experiments on \([\text{H}]\)thymidine uptake. Our results showed that DA induced cellular toxicity only at \(10^{-6} \text{ M}\), whereas the percentages of both early apoptotic and dead NK cells were never \(>28\%\) when we incubated activated NK cells with DA at \(10^{-9} \text{ M}\). Therefore, the inhibition of NK cell proliferation observed with DA at \(10^{-9} \text{ M}\) is primarily associated with an immune-modulatory function. The ability of DA to immune-regulate rather than kill NK cells is further confirmed by our experimental evidence showing that even the incubation of NK cells with either \(10^{-12} \text{ or } 10^{-15} \text{ M}\) of DA results in a statistically significant inhibition of NK cell proliferation, whereas it does not induce apoptosis or cell death compared with negative controls (Fig. 2B).

Because engagement of the D1 and D5 receptors was reported to impair the cytotoxicity of CD8+ T cells and the function/differentiation of regulatory T cells (22, 23, 38), we hypothesized that the DA-mediated suppression of NK cell division could be mediated by the engagement of D1-like DARs. These experimental hypotheses argue against the fact that resting NK cells do not constitutively express D1 and have very low levels of D5 at both cellular and transcriptional levels (Fig. 1). Hence, we analyzed the distribution of DARs in rIL-2–stimulated NK cells by detecting their transcript levels in time-course experiments. Interestingly, we found that the levels of mRNA copies of the D5 receptor increased significantly (>15-fold) in rIL-2–activated NK cells compared with resting NK cells. The upregulation of this D1-like DAR required full and prolonged cell activation, because 1 d of stimulation of NK cells with rIL-2 did not result in a significant change in DAR distribution. Similar to resting NK cells, we could not detect any D1 DAR mRNA on activated NK cells, and prolonged incubation with rIL-2 did not significantly alter the transcript levels of any of the D2-like DARs on NK cells (Fig. 2C). To demonstrate the functional relevance of the increased level of the D5 receptor following cellular stimulation, we measured the proliferation rates of rIL-2–activated NK cells preincubated with

**FIGURE 2.** Effect of DA on proliferation of rIL-2–activated NK cells. (A) Proliferation rates of rIL-2–activated NK cells incubated for 18 h with different concentrations of DA compared with rIL-2–activated NK cells cultured in the absence of DA (Mock). Data are medians of \([\text{H}]\)thymidine uptake (cpm) \((n = 6 \pm SD)\). (B) Representative flow cytometric dot plots showing the percentage of early apoptotic (Annexin V+/PI−) and dead (Annexin V+/PI+) NK cells among the lower right quadrant of each dot plot and dead (Annexin V+/PI+) cells in the lower right quadrant of each dot plot) rIL-2–activated NK cells cultured with different concentrations of DA. (C) Transcript levels of the D1, D2, D3, D4, and D5 receptors following the stimulation of NK cells with 200 IU of rIL-2 in time-course experiments. Data are presented as median fold increase \((n = 5 \pm SD)\). (D) Proliferation rates of rIL-2–activated NK cells incubated for 18 h with D1-like and D2-like DAR agonists compared with rIL-2–activated NK cells cultured alone (Mock). Data are median \([\text{H}]\)thymidine uptake (cpm) \((n = 5 \pm SD)\). *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\).
either D1-like or D2-like agonists. As expected, selective stimulation with the D2, D3, or D4 agonist did not suppress NK cell division, whereas NK cells cultured with the D1/D5 agonist SKF 38393 exhibited a significant reduction in the levels of [3H]thymidine uptake compared with control experiments (Fig. 1D). These results confirmed that the DA-mediated suppression of proliferation in activated NK cells is mediated by the engagement of the D5 receptor.

DA inhibits the production of IFN-γ by rIL-2–activated NK cells via upregulation of the D5 receptor

We then assessed whether DA is also able to affect the cytolytic potential of activated NK cells by evaluating their degree of CD107a degranulation in the presence of the conventional human erythroleukemia tumor target cell line K562 (34). Although activation with the high dose of rIL-2 significantly increased the transcript levels of D5 (Fig. 2C) in NK cells expressing even higher levels of CD56 (39), we did not detect any significant contribution of DA to a change in the percentage of CD107a+ NK cells (Supplemental Fig. 2). The lack of DA-mediated modulation of rIL-2–activated NK cell degranulation is in line with our data demonstrating that an 18-h incubation with this neurotransmitter at the above-mentioned doses did not change the surface expression of inhibiting and activating NKRs regulating NK cell cytotoxicity (data not shown).

We then proceeded to analyze whether DA plays any role in modulating the NK cell secretion of IFN-γ, an important proinflammatory cytokine that plays key roles in regulating both innate and adaptive immune responses (25, 26). Interestingly, we observed that the incubation of rIL-2–activated NK lymphocytes with different doses of DA in the presence of K562 target cells inhibited the release of IFN-γ in cell supernatants in a dose-dependent manner (Fig. 3A). Again, this phenomenon is primarily mediated by the D5 receptor, because we observed a statistically significant and dose-dependent suppression of IFN-γ release when activated NK cells cultured with DA at 10^{-9} M were preincubated with the D1-like DAR antagonist SCH 23390 (Fig. 3B). We also observed a similar DA-mediated inhibition of IFN-γ secretion using the HEK-293 target cell line (data not shown). Additionally, we showed that the incubation of rIL-2–activated NK lymphocytes with 10^{-9} and 10^{-12} M of DA significantly reduced the levels of IFN-γ mRNA in dose-dependent manner (Fig. 3C). Furthermore, the incubation of rIL-2–activated NK cells with the SKF 38393 D1-like agonist induced a statistically significant decrease in IFN-γ transcripts compared with control (Fig. 3D). Taken together, these results clearly demonstrate that DA is able to suppress the production of this inflammatory cytokine via the engagement of the D5-signaling pathway on activated NK cells.

DA-mediated posttranscriptional suppression of IFN-γ synthesis in activated NK cells is associated with the miR-29a pathway

In the context of NK cell downstream signaling following stimulation with γ-chain inflammatory cytokines like rIL-2 (40), it was demonstrated that activation of the transcription factor NF-κB selectively induces cytokine release, whereas it is dispensable for cytotoxicity (41, 42). In particular, the dimer composed of p50 and c-REL subunits within the NF-κB protein complex serves as a transcriptional repressor of miR-29a that was recently reported to negatively regulate the posttranscriptional levels of IFN-γ mRNA in both murine and human NK cells (31, 43). To disclose whether miR-29a plays any role in the DA-mediated suppression of IFN-γ expression, we measured the levels of miR-29a mRNA in rIL-2–activated NK cells cultured in the presence or absence of DA (10^{-9} and 10^{-12} M). Our results showed a significant increase in miR-29a mRNA copies in NK cells cultured with DA or

**FIGURE 3.** Effect of DA on secretion and production of IFN-γ by rIL-2–activated NK cells. (A) Amount of IFN-γ secreted by rIL-2–activated NK cells incubated for 18 h with different concentrations of DA compared with rIL-2–activated NK cells cultured in the absence of DA (Mock) (n = 5 ± SD). (B) Amount of IFN-γ secreted by rIL-2–activated NK cells cultured for 18 h with DA at 10^{-9} M and preincubated or not (Control) with either D1-like DAR antagonist in dose-dependent experiments or with D2-like DAR antagonists pooled together. Data are medians of percentages of inhibition (n = 5 ± SD). (C) Amount of IFN-γ transcripts synthesized by rIL-2–activated NK cells incubated for 18 h with different concentrations of DA compared with rIL-2–activated NK cells cultured in the absence of DA (Mock). Data are medians of fold decrease (n = 5 ± SD). (D) Amount of IFN-γ transcripts synthesized by rIL-2–activated NK cells cultured for 18 h with the D1-like DAR agonist compared with rIL-2–activated NK cells cultured alone (Mock). Data are medians of fold decrease (n = 5 ± SD). **p < 0.01, ***p < 0.001.
incubated with the D1-like DAR agonist SFK 38393 compared with control experiments (Fig. 4A, 4B). Because the suppression of miR-29a at the transcriptional level depended on the direct binding of p50 (NF-κB1) and c-REL proteins to the promoter of the miR-29a gene (31), we performed CHIP experiments to detect the amount of p50-miR-29a complexes by DNA immune-precipitation with p50 Ab in the presence or absence DA (10^{-9} M). We found a significantly decreased binding of p50 to the miR-29a promoter (Figs. 4C, 5).

**Discussion**

In addition to the conventional roles of neurotransmitters, DA, norepinephrine, and epinephrine are endowed with regulatory functions in both innate and adaptive immune responses. DA certainly plays a major role in the context of cross-talk between the nervous and immune systems, because it was found to have a great impact on the regulation of DC and T cell responses (16, 17, 22). In the current study, we identified a novel immune-modulatory function of DA that is able to switch off NK cell effector functions by inhibiting cellular proliferation and suppressing synthesis of proinflammatory cytokines. This DA-mediated control of NK cell functions could represent a checkpoint that controls NK cell reactivity in the early phases of innate immune responses to avoid the establishment of chronic and potentially dangerous inflammatory processes at tissue sites (Fig. 5).

In this article, we show that freshly purified NK cells preferentially express D2-like DARs, with the D4 receptor being dominant. However, in contrast to what was described recently for murine NK cells (30), we did not observe any impact of DA on effector functions of resting NK cells. In contrast, human NK cells become susceptible to immune-regulatory functions exerted by DA only in response to potent and prolonged cellular activation. Indeed, we demonstrate that the stimulation of NK cells with high doses of rIL-2 for 5 d induces a significant upregulation of D5, an inhibitory receptor belonging to the family of D1-like DARs that induces NK cell division and production of IFN-γ following its binding with DA. Immune cells are generally exposed to DA that is present in the human plasma at physiological concentrations (10^{-10}–10^{-12} M) and is directly secreted by the dopaminergic innervation of primary and secondary lymphoid organs (5, 23). The fact that resting NK lymphocytes, although constitutively expressing D2-like DARs, do not respond to the concentrations of DA physiologically present in tissues and plasma reflects the intrinsic nature of these cells. Indeed, NK lymphocytes can be immediately activated by a variety of cytokines and chemokines secreted at sites of inflammation to exert the killing of viral-infected or tumor-transformed cells target or to establish cellular networks linking innate and adaptive immunity (25–27, 44–49). Instead, NK cells need mechanisms that control and regulate their level of activation and effector function. Our data indicate that DA plays such role, at least in part. Furthermore, we found that DA is able to suppress the production of IFN-γ in experimental settings that used both K562 and HEK-293 cell lines, whereas it did not have any effect on the killing of these two targets expressing different patterns of ligands for NKRs (50, 51). These experimental findings demonstrate the intrinsic immune modulatory ability of this neurotransmitter that is able to modulate activated NK cell responses regardless of the interactions of NKRs with their putative ligands. It was previously reported in murine NK cells that the selective activation of D1-like DARs increases cytotoxicity, whereas the engagement of D2-like DARs dampens killing activities (30). We show that this is not the case for human NK cells, and this functional dichotomy between mouse and human highlights once more the complex and heterogeneous immune modulations exerted by DA.

It was reported that DA-mediated inhibition of human CD4+ and CD8+ cell proliferation requires engagement of the D1 receptor via the intracellular secondary messenger CAMP (23, 37), which is known as one of the main inhibitors of lymphocyte growth (52, 53). Although NK cells do not express the D1 receptor, we

**FIGURE 4.** Effect of DA on NF-κB– and miR-29a–signaling pathways in rIL-2–activated NK cells. (A) Amount of IFN-γ and miR-29a transcripts synthetized by rIL-2–activated NK cells incubated for 18 h with DA at 10^{-9} and 10^{-12} M compared with rIL-2–activated NK cells cultured in the absence of DA (Mock). Data are medians of fold change (n = 5 ± SD). (B) Amount of miR-29a transcripts synthetized by rIL-2–activated NK cells cultured for 18 h with the D1-like DAR agonist compared with rIL-2–activated NK cells cultured alone (Mock). Data are medians of fold increase (n = 5 ± SD). (C) CHIP arrays showing the amount of p50-miR-29a DNA complexes in the immune precipitates of rIL-2–activated NK cells incubated for 18 h with DA at 10^{-9} M compared with rIL-2–activated NK cells cultured in the absence of DA (Mock) (left panel). Data were normalized to GAPDH gene expression in input samples and are presented as medians of fold change (n = 5 ± SD). The agarose gel shows one representative CHIP amplification product (lower left panel) and the sheared isolated chromatin (right panel) at lengths of 500 and 1000 bp. p < 0.05, **p < 0.01, ***p < 0.001.
DOPAMINE INHIBITS NK CELL FUNCTIONS

FIGURE 5. Dopaminergic signaling pathways associated with regulation of NK cell effector functions. The engagement of IL-2R in NK cells is physiologically coupled with the downstream signaling mediated by the transcription factor NF-κB, whose activation allows the dimer p50/cREL to suppress the de novo synthesis of miR-29a and, thus, induce IFN-γ synthesis. The stimulation of the D5 receptor with DA following a persistent and potent IL-2 activation of NK cells inhibits the binding of the p50 subunit of the NF-κB complex to the miR-29a promoter. Therefore, the lack of repressor activity of the p50/cREL dimer remarkably increases the de novo synthesis of miR-29a, which, in turn, blocks the production of IFN-γ. Given the well-established bidirectional interactions of NK lymphocytes with DCs, monocyte/macrophages, and regulatory T cells (Tregs), regulatory DA-mediated paracrine loops also can contribute to control and shape NK cell effector functions in the presence of persistent inflammatory stimuli at mucosal surfaces or tissues sites.

Demonstrate in this study that the DA-mediated inhibition of DNA synthesis in these lymphocytes occurs via the D5 receptor, which also was reported to increase intracellular levels of cAMP (18–20). These results are in line with other studies showing that the incubation of NK cells with molecules able to increase the intracellular level of cAMP (i.e., forskolin or dibutyryl cAMP) also inhibits the proliferation of these lymphocytes (54). It is well known that RIL-2 is a potent inducer of NK cell proliferation and production of IFN-γ, a cytokine that play a key role in the immune responses against viruses, intracellular bacteria, and tumors (27, 55, 56). Multiple transcription factors, such as Stat4, NF-κB, T-box, and T-bet, are involved in the regulation of IFN-γ production by NK cells upon engagement of cell surface molecules with their putative ligands or following stimulation with several cytokines (55). In line with a previous report (31), our data show that the interaction between miR-29a promoter and NF-κB is also controlled by engagement of the DA pathway in activated NK cells (Fig. 4C). Indeed, we detected a relatively low, but persistent, inverse correlation between miR-29a and IFN-γ expression in NK cells incubated with either DA or D1-like DAR (Fig. 4A, 4B). This indicates that miR-29a is likely involved in DA-mediated regulation of IFN-γ gene expression in activated NK cells. However, the biological relevance of these changes still needs to be investigated and fully elucidated. Indeed, we have to take into account that QPCR analyses of the specific mature microRNA might be underestimated, considering that the inherent stability of the specific microRNA may be affected by cell activity and stimulation (57, 58). Furthermore, the effect of DA on miR-29a regulation also may be the consequence of other molecular mechanisms. Further investigations are required to fully understand the mechanisms driving the DA-inducible pathway of IFN-γ inhibition in human NK cells.

Our data showing the DA-mediated suppression of IFN-γ via NF-κB transcription factor are in line with the reported inhibitory action of cAMP on NF-κB-mediated signaling in immune cell functions (59–61). In this regard, the administration of L-DOPA in murine models was shown to decrease the frequency of IFN-γ-producing cells in the spleen (62) and T cell compartment (12), indicating that IFN-γ gene regulation is an important immune target for this neurotransmitter.

Other than by the levels of DA physiologically present in human plasma or released by the dopaminergic innervation of lymphoid organs (16, 17), the ability of DA to modulate NK cell effector functions can occur through immunologic paracrine regulatory loops, because the activation or differentiation of several immune cells was shown to either induce or increase their capacity to synthesize, store, and release DA (10, 16, 17, 22, 63). Given the well-established bidirectional interactions between NK lymphocytes and APCs, such as DCs and monocyte/macrophages (44–46, 48), it is conceivable to hypothesize that the increased ability of these professional APCs to produce DA in response to Ag recognition and following cellular activation might serve as a regulatory mechanism that controls and shapes NK cell effector functions. However, further investigations are required to confirm that DA-mediated immunologic cross-talk is involved in the control of NK cell responses.

Our discovery of DA’s ability to regulate NK cell proliferation and production of IFN-γ opens new avenues to better understand the pathogenesis of neurodegenerative diseases, such as Parkinson’s disease (PD). Indeed, new lines of evidence support the emerging hypothesis that inflammation plays a critical role in the early phases of neurodegeneration, because a specific genetic susceptibility observed in PD patients activates immune pathways and inflammatory reactions (64). Indeed, regular users of non-steroidal anti-inflammatory drugs are at decreased risk for developing PD (65). Neuroinflammation in PD includes activation of microglia, increased secretion of cytokines and PGs, activation complement, and circulating NK cells (66). In particular, the levels of IFN-γ are higher in the serum of PD patients compared with healthy donors, and freshly purified circulating NK cells from PD patients were reported to spontaneously release greater amount of IFN-γ compared with healthy volunteers (67, 68). The pathologic homing of NK cells to the central nervous system might help to explain the higher amount of inflammatory cytokines observed in...
the cerebrospinal fluid and striatum of PD patients with impaired dopaminergic transmission (38, 66, 69–72). Therefore, the lack of or impaired D5-mediated suppression of NK cell proliferation and secretion of IFN-γ due to a decreased engagement of DA pathway during the course of PD theoretically can explain the chronic state of activation of these innate lymphocytes.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1
NK cell enrichment
Flow cytometric dot plot graphs from one representative healthy donor showing total NK cells either within total PBMCs (upper line) or after enrichment by negative magnetic bead depletion (middle line). The third line shows the overlap of two flow cytometric staining showed above. NK cells are gated within CD14<sup>neg</sup> lymphocyte gate and labeled as CD56<sup>pos</sup>/CD19<sup>neg</sup> or CD56<sup>pos</sup>/CD3<sup>neg</sup> lymphocytes. An appropriate negative PC5 isotype control was use for detecting CD56<sup>pos</sup> NK cells.

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Supplemental Figure 1
Supplemental Figure 2
CD107a degranulation of freshly purified and rIL-2 activated NK cells
A, Flow cytometric dot plot graphs from a representative healthy donor showing the percentages of freshly purified (left panel) and rIL-2 activated (right panel) CD3<sup>neg</sup>/CD14<sup>neg</sup>/CD20<sup>neg</sup> CD16<sup>pos</sup>/CD56<sup>pos</sup>/CD107a<sup>pos</sup> NK cells in response to incubation with K562 cell target for 3 hours at a 1:1 ratio (34). The percentages of CD107a<sup>pos</sup> NK cells are gated in the upper quadrants of each dot plot.
B, Summary graphs of statistical histogram bars showing the percentages of freshly purified (left panel) and rIL-2 activated (right panel) CD3<sup>neg</sup>/CD14<sup>neg</sup>/CD20<sup>neg</sup> CD16<sup>pos</sup>/CD56<sup>pos</sup>/CD107a<sup>pos</sup> NK cells either in the absence (untreated) or in the presence of a prior incubation with DA at different concentrations. Data are presented as medians (N=6± SD).

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Supplemental Figure 2
Supplemental Figure 2

CD107α degranulation of freshly purified and rIL-2 activated NK cells

A, Flow cytometric dot plot graphs from a representative healthy donor showing the percentages of freshly purified (left panel) and rIL-2 activated (right panel) CD3\(^{neg}/\)CD14\(^{neg}/\)CD20\(^{neg}/\)CD16\(^{pos}/\)CD56\(^{pos}/\)CD107\(^{a pos}\) NK cells in response to incubation with K562 cell target for 3 hours at 1:1 ratio (34). The percentages of CD107\(^{a pos}\) NK cells are gated in the upper quadrants of each dot plot.

B, Summary graphs of statistical histogram bars showing the percentages of freshly purified (left panel) and rIL-2 activated (right panel) CD3\(^{neg}/\)CD14\(^{neg}/\)CD20\(^{neg}/\)CD16\(^{pos}/\)CD56\(^{pos}/\)CD107\(^{a pos}\) NK cells either in the absence (untreated) or in the presence of a prior incubation with DA at different concentrations. Data are presented as medians (N=6± SD).

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Supplemental Figure 2