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Research Article

Modulation of natural killer cell cytotoxicity and cytokine release by the drug glatiramer acetate

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Abstract. Glatiramer acetate (GA or Copaxone) is a drug used to treat experimental autoimmune encephalomyelitis in mice and multiple sclerosis in human. Here, we describe a new mechanism of action for this drug. GA enhanced the cytolysis of human NK cells against autologous and allogeneic immature and mature monocyte-derived dendritic cells (DCs). This drug reduced the percentages of mature DCs expressing CD80, CD83, HLA-DR or HLA-I. In contrast, it did not modulate the percentages of NK cells expressing NKG2D, NKp30, or NKp44. Nonetheless, anti-NKp30 or anti-CD86 inhibited GA-enhanced human NK cell lysis of immature DCs. Hence, CD86, and NKp30 are important for NK cell lysis of immature DCs, whereas CD80, CD83, HLA-DR and HLA-I are important for the lysis of mature DCs when GA is used as a stimulus. Further, GA inhibited the release of IFN-γ 24 h but increased the release of TNF-α 48 h after incubation with NK cells.

Keywords. Glatiramer acetate, NK cells, dendritic cells, cytotoxicity, autoimmunity.

Introduction

Glatiramer acetate (GA; commercial name Copaxone) is a synthetic compound made up of four amino acids (Glu, Ala, Lys, Tyr) that are found in myelin. It prevents the incidence of experimental autoimmune encephalomyelitis (EAE) in animals, reduces relapses in patients with multiple sclerosis (MS) [1], prolongs skin graft survival, and inhibits graft versus host (GvH) disease [2]. In a six-year trial, MS patients who received GA had a steady decline in disease relapses with neurological improvement when compared to patients receiving placebo [3]. This drug is thought to mediate its beneficial effects by induction of GA-specific T helper (Th) 2 cells. IFN-γ, a Th1 cytokine production is reduced and the ratio of IL-4/IFN-γ significantly increased in MS patients treated with GA [4], suggesting a shift from Th1 to Th2 after therapy with GA. In addition, GA induces the activation of Treg cells [5] and inhibits the secretion of IL-12p70 after stimulating dendritic cells (DCs) with CD40L, resulting in increased IL-4 and decreased IFN-γ release by T cells stimulated with GA-pretreated DCs [6].

Although the mechanisms of shifting the immune system toward Th1 are not entirely clear, Weber et al. demonstrated that incubating monocytes *in vitro* with GA renders these cells less responsive to multiple stimuli, including toll-like receptors (TLRs) ligands and inflammatory cytokines such as IFN-γ and GM-CSF [7]. They also demonstrated that monocytes from GA-treated MS patients respond with lower efficiency to stimulation with LPS. In

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addition, monocytes isolated from GA-dosed MS patients secrete high amounts of the anti-inflammatory cytokine IL-10 and less of the inflammatory cytokine IL-12 [8]. This switch toward type 2 antigen presenting cells (APCs) is confirmed by Weber et al. who demonstrated that administration of GA into mice with EAE favors the activation of type 2 monocytes which induce naïve T cells to become Th2, as well as activating T regulatory (Treg) cells [9]. Also, activation of type 2 monocytes is corroborated with reduced inflammation in the central nervous system of GA-treated EAE mice (9).

NK cells are anti-tumor and anti-microbial effectors [10, 11]. In the blood circulation, human NK cells are classified into two major subsets; regulatory cells expressing CD56 but not CD16 known as $CD16^{-}$ $CD56^{+/high}$, and cytolytic cells expressing CD16 and low CD56 known as $CD16^{+}CD56^{-/low}$ [11]. Depletion of NK cells before immunization of sensitive mice with myelin oligodendrocyte glycoprotein (MOG_{35-55}) peptide resulted in clinically more severe relapsing EAE [12]. In CX_3CR1 deficient mice, NK cell recruitment into the CNS of animals with EAE is impaired, corroborated with increasing the severity of the disease [13]. However, these results contradict other studies showing that NK cells exacerbate rather than ameliorate EAE. For example, localized IL-12 by astrocytes promotes the spontaneous development of NK cells which enhance Th1 cell activity and cytokine secretion [14]. Also, increased IL-18 production during the primary injection of MOG_{35-55} results in increased production of IFN-γ secreted by NK cells, promoting autoreactive Th1 responses, whereas an impaired capacity of NK cells to release IFN-γ is found to be the major mechanism underlying resistance to EAE [15].

We recently reported that administration of GA into mice suffering from EAE ameliorates the EAE clinical scores corroborated with isolating NK cells that lyse both immature and mature DCs (16). In the present communication we examined whether GA influences human NK cells to lyse syngeneic or allogeneic immature and mature monocyte-derived DCs *ex vivo,* as well as its effect on cytokines and chemokines release by NK cells. We report that NK cells exposed to GA lyse both immature and mature DCs regardless whether these cells are isolated from autologous or allogeneic donors. We also report that IFN-γ release by NK cells is reduced 24 h after stimulation with GA, whereas TNF-α is increased 48 h later.

Materials and methods

Preparation of human cells. Peripheral blood cells were obtained from healthy volunteers (Ullevål Hospital, Oslo, Norway) and centrifuged over histopaque gradients (Sigma-Aldrich, Oslo, Norway). Human IL-2-activated NK cells were prepared from 50 x 10^6/ml mononuclear cells separated using histopaque (Sigma Aldrich). The cells were purified using EasySep negative selection NK cell enrichment kit (StemCell Technologies SARL, Grenoble, France), which removes CD3, CD4, CD19, CD20, CD36, CD66b, CD123, HLA-DR, and glycophorin A positive cells, leaving NK cells intact. The pure NK cells were incubated at 1 x 10^6 cells/ml with 200 U/ml IL-2 at 37 ^{0}C in a 5% CO_2 incubator for five to seven days. Viability was more than 90% after the incubation period as determined by trypan blue exclusion test.

Human monocytes were isolated using RosetteSep human monocyte enrichment cocktail (Stem Cell Techniques). $CD14^+$ cells collected after this isolation procedure were cultured in sterile Petri dishes at 2 x 10^6/ml with 6 ng/ml IL-4 and 25 ng/ml rhGM-CSF (ImmunoTools, Friesoythe, Germany), for 5 days at 37 °C to generate immature (i) DCs. Mature DCs (mDCs) were generated by adding 1 μg/ml LPS (Sigma-Aldrich, Oslo, Norway) to the cultures of iDCs for 2 days, as described [17]. More than 90% of the cells were viable after the culture period.

NK cell cytotoxicity assay. This method has been described recently [18]. Human immature and mature dendritic cells were used as targets. Target cells were incubated at 1 x 10^6 cells/ml with 5 μg/ml calcein-AM (Teflabs, Austin TX, USA) for 1 h at 37 °C. After this, target cells (10 000/well) and effector cells were plated onto 96-well plates at the indicated effector : target (E:T) cell ratios in triplicate. For lysis of human DCs, these cells were incubated with activated NK cells in the absence or the presence of various concentrations of GA, 10 μg/ml of anti-NKG2D, anti-NKp33, anti-NKp46, anti-CD80, anti-CD83, anti-CD86 (all from Beckton-Dickinson, San Diego, CA), anti-HLA-I, anti-HLA-DR (ImmunoTools), or anti-CCR7 (R&D systems, Minneapolis MN, USA). The plates were spun down at 500 rpm for 5 min and incubated for 4 h at 37 °C. After incubation, the cells were centrifuged, the supernatants removed and PBS added to each well. The fluorescence intensity of the calcein AM-loaded cells was measured in a BioTek FLX TBI plate reader, using 485/528 nm fluorescence filters. Percentage cytotoxicity was calculated as described [18].

Flow cytometric analysis. To stain NK cells with antibodies for various NK cell receptors, these cells

were incubated with GA for 4 h, washed and stained with 5 μg/ml PE-conjugated mouse anti-human CD69 (ImmunoTools), 1 μg/ml PE-conjugated mouse anti-human NKp30 (CD337), 1 μg/ml PE-conjugated mouse anti-human NKp44 (CD336), 1 μg/ml PE-conjugated mouse anti-human NKG2D (CD314) or control PE-conjugated mouse IgG1 (All antibodies were from Beckton-Dickinson Pharmingen), for 45 min at 4 °C. The cells were washed twice, and examined in the flow cytometer (FACSCalibur, Becton Dickinson Biosciences, San Jose, CA). Gating was performed according to the isotype control.

Immature and mature DCs were incubated with GA for 4 h, washed and incubated in a 96-well plate (v-bottom, $2X10^5$ cells per well). They were washed again and resuspended in PBS buffer containing 0.1 % sodium azide. DCs were labeled with 1 μg/ml FITC-conjugated mouse anti-human CD80, FITC-conjugated mouse anti-human CD83, FITC-conjugated mouse anti-human CD86 (BD Biosciences Pharmingen, San Diego CA, USA), FITC-conjugated mouse anti-human HLA-class I, FITC-conjugated mouse anti-human HLA-DR (ImmunoTools), FITC-conjugated mouse anti-CCR7 (R&D systems) or FITC-conjugated mouse IgG (Beckton-Dickinson Pharmingen) as a control. The cells were washed twice, and examined in the flow cytometer. Gating was done according to the isotype control.

Detection of cytokines and chemokines release utilizing the ELISArray kits. NK cells were incubated at a cell concentration of 1 x 10^6/ml either with vehicle or with 1 or 40 μg/ml GA for 24 or 48 h. After incubation, the cells were harvested (no wash step) and the cell suspensions were centrifuged at 1000 x g for 10 min before the supernatants were collected. The samples from 24 h incubation were kept at -70 °C until testing. The samples from 48 h incubation were kept on ice until they were added to the ELISArray plates. Detection of the levels of various cytokines and chemokines was carried out utilizing the Multi-Analyte ELISArray Kit (SA Biosciences MD, USA) as described by the manufacturer's user manual. The kit analyzes the concentrations of IL-1β, IL-4, IL-6, IL-10, IL-12, IL-17A, IFN-γ, TNF-α, TGF-β1, MCP-1, MIP-1α, and MIP-1β. Negative and positive controls supplied by the kits were also included.

Statistical analysis. Significant values were generated using the Student *t*-test. A P value < 0.05 was considered to be statistically significant. Geometric mean of fluorescence intensity was calculated using a program provided with the FACSCalibur (Beckton-Dickinson).

Results

GA enhances human NK cell cytolysis of DCs. The effect of GA on the ability of human NK cells to lyse DCs was investigated, since it has been shown that DCs are involved in EAE [19, 20]. For this purpose, we first used immature dendritic cells (iDCs) as targets. Addition of 1, 10, 20 and 40 μg/ml GA significantly enhanced the cytolysis of NK cells (used at 10:1 and 5:1 E:T cell ratios) against autologous iDCs (Fig. 1A), or allogeneic iDCs (Fig. 1B). Almost similar results were observed with mature dendritic cells (mDCs), regardless of whether syngeneic mDCs (Fig. 1C), or allogeneic mDCs (Fig. 1D) were used as targets for NK cells.

Attributes of GA target molecules on NK cells. To determine which receptor might be involved in GA enhancement of human NK cell lysis of DCs, we incubated various antibodies to the C-type lectin NKG2D, or the natural cytotoxicity receptors NKp30 and NKp44 with the cultures of NK cells and DCs in the presence of GA. The results show that GA enhanced NK cell lysis of syngeneic iDCs ($P < 0.001$ as compared to the control, Fig. 2A), or syngeneic mDCs ($P < 0.05$ as compared to the control, Fig. 2B). In addition, only anti-NKp30 but not anti-NKG2D or anti-NKp44, significantly inhibited the enhancement of NK cell lysis of iDCs induced by GA ($P < 0.008$ as compared to cells cultured in the presence of GA, Fig. 2A). Antibody against NKp44 decreased GA effect but this did not reach statistical significance. On the other hand, none of the antibodies used affected GA-enhancement of NK cell lysis of mDCs (Fig. 2B). To correlate these effects with the expression of NK cell receptors, we examined the surface expression of these molecules on NK cells. In three separate experiments the addition of 1 or 40 μg/ml GA to NK cells did not significantly affect the percentages of cells expressing NKG2D, NKp30, NKp44, or the mean fluorescence intensity (MFI) of these receptors as measured by flow cytometric analysis (Table 1). It should be noted that pre-treatment with both concentrations of GA for 4 h reduced the MFI of NKG2D, NKp30, and NKp44, but this reduction did not reach statistical significance in cells isolated from three different donors.

Attributes of GA target molecules on iDCs. The effect of various molecules expressed on the surface of iDCs in GA-mediated enhancement of NK cell lysis of iDCs was the subject of the next set of experiments. Antibodies to CD80, CD83, CD86, HLA-DR and CCR7 were incubated with NK cells and iDCs in the presence of 40 μg/ml GA. GA significantly enhanced

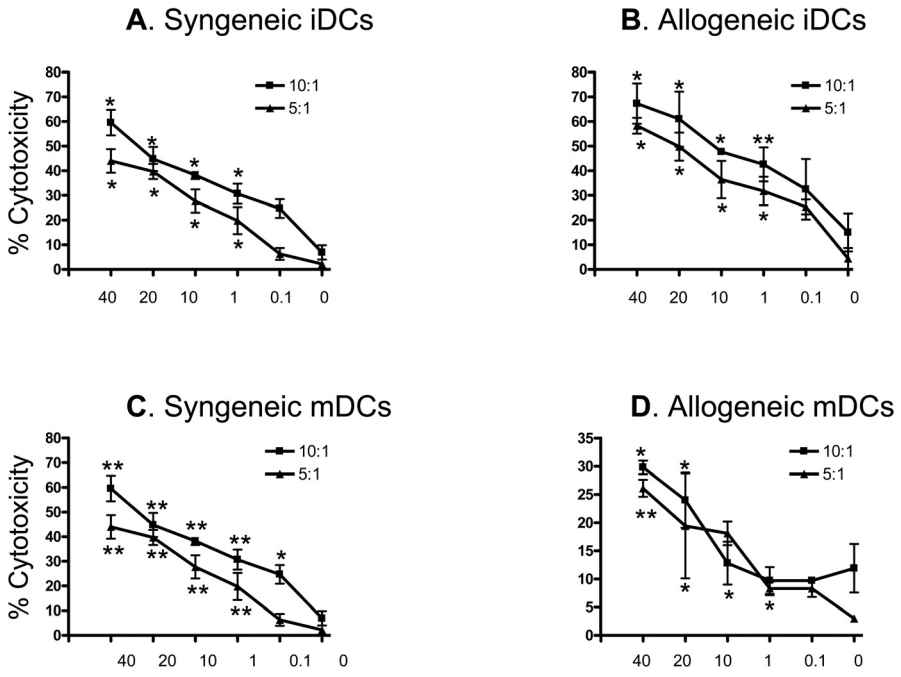

Figure 1. GA enhances human NK cells lysis of dendritic cells (DCs). (*A*) NK cells were incubated with syngeneic iDCs at 10:1 and 5:1 E:T cell ratios, in the absence (0) or the presence of various concentrations (0.1, 1, 10, 20 or 40 μg/ml) of GA. (*B*) This is similar to panel (*A*) except that allogeneic iDCs were used as targets. Significant values as compared to the control are shown on top of the columns; stars above show differences between the control and those where GA was added (NK plus DCs used at 10:1 E:T ratio), and those below show the differences among the same cultures but when 5:1 E:T cell ratio was used. (*C*) NK cells were incubated with syngeneic mature DCs (mDCs) at 10:1 and 5:1 E:T cell ratios, in the absence (0) or the presence of various concentrations (0.1, 1, 10, 20 or 40 μg/ml) of GA. (*D*) This is similar to panel (*C*) except that allogeneic mDCs were used as targets. Significant values as compared to the control are shown on top of the columns; stars above show differences between the control and those where GA was added (NK plus DCs used at 10:1 E:T ratio), whereas those below show the differences among the same cultures but when 5:1 E:T cell ratio was used. * = $p < 0.05$, and ** = $p < 0.001$, as compared to the controls. Results shown are from four different donors.

Table 1. Percentages of NK cells expressing NK cytotoxicity receptors and their mean fluorescence intensity.

Treatment	NKG2D				NKp30				NKp44			
	%[a]	P[b]	MFI[c]	P[d]	%[a]	P[b]	MFI[c]	P[d]	%[a]	P[b]	MFI[c]	P[d]
No Treatment	69 ± 10		56 ± 7		77 ± 11		85 ± 14		45 ± 8	NS	44 ± 9	
1 μg/ml GA	68 ± 13	NS	43 ± 2	NS	76 ± 13	NS	74 ± 12	NS	39 ± 13	NS	36 ± 3	NS
40 μg/ml GA	67 ± 9	NS	44 ± 6	NS	76 ± 12	NS	75 ± 9	NS	48 ± 15	NS	38 ± 4	NS

NK cells were either left untreated or were incubated with 1 or 40 μg/ml GA for 4 h. These cells were washed, stained with antibodies and then examined for the expression of NKG2D, NKp30 and NKp44.
[a] Mean ± SEM of percentages of positive cells isolated from three different donors.
[b] P values compare the percentages of positive cells between GA-treated and untreated cells.
[c] Mean fluorescence intensity (MFI) of cells obtained from three different donors.
[d] P values compare the MFI of the expression of the receptor among GA-treated and untreated cells. NS = not significant.

NK cell lysis of syngeneic iDCs ($P < 0.001$ as compared to the control, Fig. 3). This activity was significantly inhibited by the addition of anti-CD86 antibody ($P < 0.01$ as compared to the cultures of cells incubated with GA only, Fig. 3). Anti-CD80, anti-CD83, and anti-HLA-I tend to decrease the effect of GA, but this did not reach statistical significance in five separate experiments. To demonstrate whether GA might affect the percentages of iDCs carrying these molecules, these cells were incubated with 1 or 40 μg/ml GA for 4 h. The results indicate that 1 or 40 μg/ml GA did not affect the expression of CD80, CD83, CD86, HLA-DR or the chemokine receptor CCR7 on the surface of iDCs (Fig. 4). This was reproduced in four separate experiments done on cells isolated from four different donors where no effect of GA was noticed on the percentages of iDCs expressing CD80, CD83, CD86, HLA-DR and CCR7 molecules, or their MFI (Table 2). However, a significant reduction in the MFI of HLA-DR was observed after

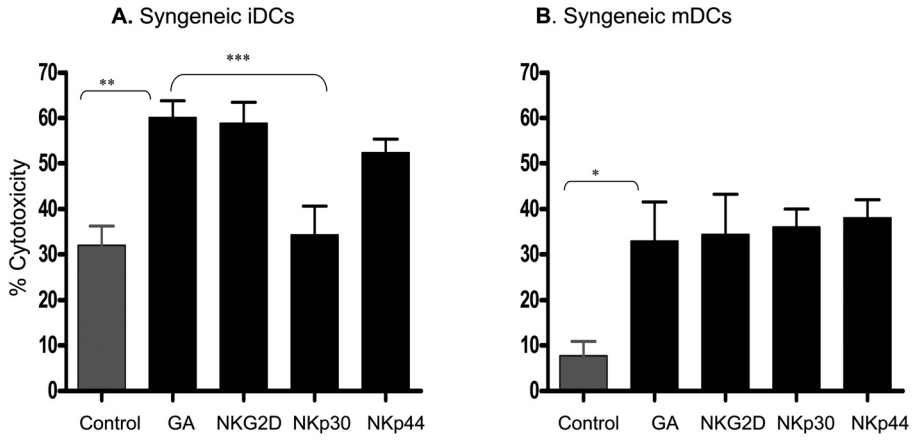

Figure 2. Effects of NK cell cytotoxicity receptors on GA-enhanced NK cell lysis of DCs. (*A*) Syngeneic iDCs were incubated with NK cells in the absence (Control) or the presence of 40 μg/ml GA. In other cultures, 10 μg/ml of anti-NKG2D, anti-NKp30 or anti-NKp44 was also added. (*B*) This is similar to (*A*) except that syngeneic mDCs were used as targets. P values compare differences between control cultures (grey columns) and those where GA was added, or between cultures where GA was added in the absence or the presence of anti-NKp30. * = $p < 0.05$, ** = $p < 0.001$, and *** = $p < 0.008$. The results show means ± SEM of five different experiments in panel (*A*) and three different experiments in panel (*B*).

Table 2. Percentages of immature dendritic cells expressing various surface receptors and their mean fluorescence intensity before and after incubation with GA.

Treatment	CD80				CD83				CD86			
	%[a]	P[b]	MFI[c]	P[d]	%[a]	P[b]	MFI[c]	P[d]	%[a]	P[b]	MFI[c]	P[d]
No Treatment	12±6		7±2		5±0.1		6±0.3		73±9		24±3	
1 μg/ml GA	19±12	NS	11±1	NS	9±3	NS	8±1	NS	86±12	NS	32±4	NS
40 μg/ml GA	22±15	NS	8±2	NS	10±6	NS	8±2	NS	85 ±12	NS	26±4	NS

Table 2. *(continued)*

Treatment	HLA-DR				CCR7			
	%[b]	P[b]	MFI[c]	P[d]	%[a]	P[b]	MFI[c]	P[d]
No Treatment	41±2		25±4		0.2±0.1		6±1	
1 μg/ml GA	42±21	NS	23±2	NS	0.6±0.2	NS	6±2	NS
40 μg/ml GA	42±11	NS	12±2	0.02	0.1±0.0	NS	6±1	NS

Immature DCs were either left untreated or were incubated with 1 or 40 μg/ml GA for 4 h. These cells were washed, stained with antibodies and then examined for the expression CD80, CD83, CD86, HLA-DR or CCR7 surface molecules.
[a] Mean ± SEM of percentages of positive cells isolated from four different donors.
[b] P values compare the percentages of positive cells between GA-treated and untreated cells.
[c] Mean fluorescence intensity (MFI) of cells obtained from three different donors.
[d] P values compare the MFI of the expression of receptor molecules among GA-treated and untreated cells.
NS = not significant.

incubating these cells with 40 μg/ml GA for 4 h (P 0.02, Table 2, also see Fig. 4).

Attributes of GA target molecules on mDCs. Further, we examined the effect of GA on various molecules in mDCs that may be involved in the cross-talk among NK cells and mDCs. Addition of GA enhanced NK cell lysis of mDCs ($P < 0.001$ as compared to the control, Fig. 5). None of the antibodies used, i.e. anti-CD80, anti-CD83, anti-CD86, anti-HLA-I, anti-HLA-DR or anti-CCR7 affected this activity of GA (Fig. 5). Flow cytometric analysis shows that 40 μg/ml of GA significantly reduced the numbers of mDCs expressing CD80 or CD83 (P 0.03 or P 0.02, respectively as compared to the control expression, Table 3). The percentages of mDCs expressing HLA-DR were also significantly reduced upon treatment with 40 μg/ml of GA (P 0.04 as compared to the control, Table 3). Moreover, the 40 μg/ml of GA significantly reduced the MFI of HLA-DR on the surface of these cells (P

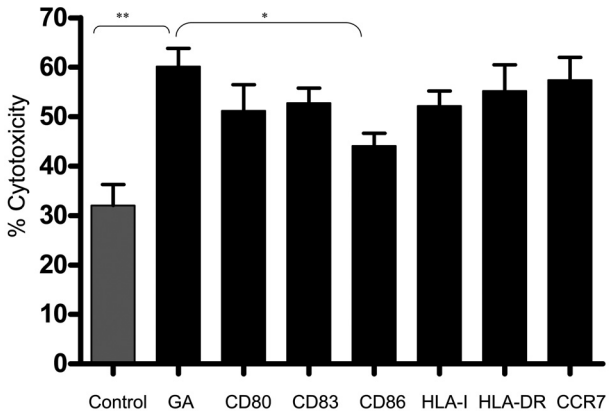

Figure 3. Effects of incubation with co-stimulatory and other molecules on GA-induced enhancement of the cytolysis of iDCs by NK cells. Immature DCs were incubated in the absence (Control) or the presence of 40 μg/ml GA. In other cultures 10 μg/ml anti-CD80, anti-CD83, anti-CD86, anti-HLA-I, anti-HLA-DR and anti-CCR7 were added. P values compare differences among control cultures and those where GA was added, or between cultures where GA was added in the absence or the presence of anti-CD86. * = $p < 0.01$, and **=$p<0.001$. Results shown are from five different donors.

GA inhibits the release of IFN-γ but increases TNF-α secretion from NK cells. Finally, we measured the release of various cytokines and chemokines from activated NK cells either left intact or incubated with 1 or 40 μg/ml of GA for 24 or 48 h. Results in Figure 6 demonstrate that NK cells secreted low amounts of IL-1β, IL-4, IL-6, IL-10, IL-12 or the chemokine MCP-1, 24 or 48 h after culture. There was low-moderate release of IL-17, TNF-α or TGF-β, but high release of IFN-γ after 24 h, and the chemokines MIP-1α and MIP-1β after 24 or 48 h of culture. Among all the cytokines and chemokines tested, 1 or 40 μg/ml GA reduced the release of only IFN-γ 24 h after culture ($P < 0.05$ for both concentrations when compared to untreated cells). Although the 40 μg/ml concentration of GA significantly increased the release of TNF-α 48 h after culture ($P < 0.003$, as compared to untreated cells), the level of this release was not impressive (Fig. 6).

Table 3. Percentages of mature dendritic cells expressing various surface receptors and their mean fluorescence intensity before and after incubation with GA.

Treatment	CD80				CD83				CD86			
	%[a]	P[b]	MFI[c]	P[d]	%[a]	P[b]	MFI[c]	P[d]	%[a]	P[b]	MFI[c]	P[d]
No Treatment	77±4		36±7		26±5		15±4		88±8	NS	126±25	
1 μg/ml GA	73±2	NS	39±7	NS	11±6	NS	17±5	NS	84±4	NS	123±22	NS
40 μg/ml GA	57±7	0.03	37±6	NS	6±5	0.02	15±4	NS	80±2	NS	101±15	NS

Table 3. *(continued)*

Treatment	HLA-DR				HLA-I				CCR7			
	%[a]	P[b]	MFI[c]	P[d]	%[a]	P[b]	MFI[c]	P[d]	%[a]	P[b]	MFI[c]	P[d]
No Treatment	90±1		80±3		93±1		57 ±18		15±5		9 ±2	
1 μg/ml GA	90±2	NS	88±10	NS	81±5	0.02	49±2	NS	12±4	NS	9±2	NS
40 μg/ml GA	42±18	0.04	18±10	0.004	78±1	0.001	46±15	0.006	6±2	NS	8±2	NS

Mature DCs were either left untreated or were incubated with 1 or 40 μg/ml GA for 4 h. These cells were washed, stained with antibodies and then examined for the expression CD80, CD83, CD86, HLA-DR, HLA-I or CCR7 surface molecules.
[a] Mean ± SEM of percentages of positive cells isolated from five-six different donors.
[b] P values compare the percentages of positive cells between GA-treated and untreated cells.
[c] Mean fluorescence intensity (MFI) of cells obtained from three different donors.
[d] P values compare the MFI of the expression of receptor molecules among GA-treated and untreated cells.
NS = not significant.

0.004, Table 3). Interestingly, 1 or 40 μg/ml of GA reduced the percentages of mDCs expressing HLA-I (P 0.02 and P 0.001, respectively, Table 3). The 40 μg/ml dose also reduced the MFI of HLA-I (P 0.006, Table 3). In contrast, no effect on the expression of CD86 or CCR7 was observed after incubating mDCs with GA.

Discussion

GA ameliorates MS and increases the period of remission in MS patients as well as reducing the EAE clinical scores in mice affected with this disease [1, 3, 4, 7, 8, 9, 16]. The major mechanism attributed to the effect of GA is shifting the immune system from an inflammatory Th1 to an anti-inflammatory Th2 type

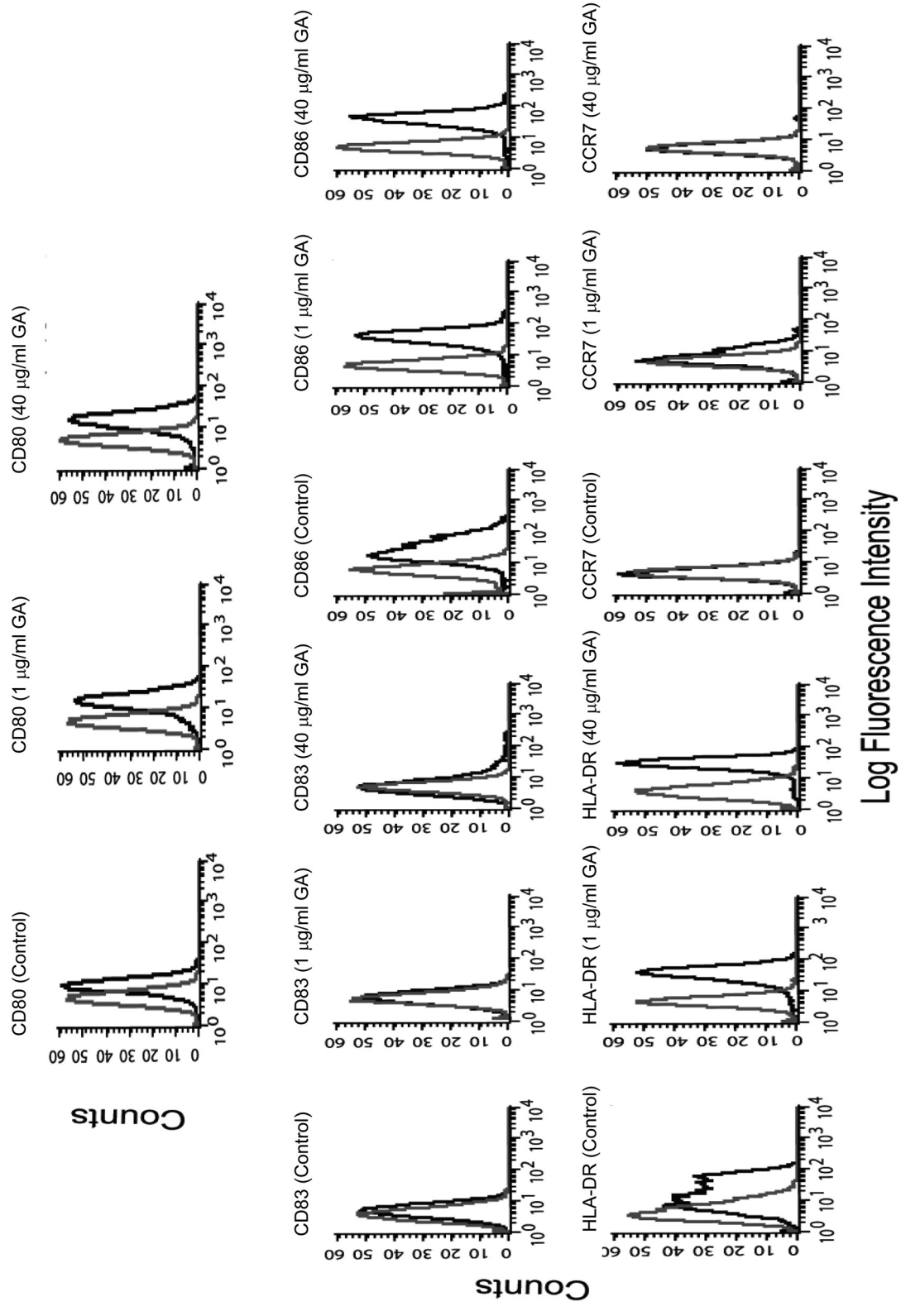

Figure 4. Expression of various molecules on the surface of iDCs before and after incubation with GA. Immature DCs were either left untreated or were incubated with 1 or 40 μg/ml GA for 4 h. These cells were washed, stained with various antibodies and then examined for the expression CD80, CD83, CD86, HLA-DR or CCR7 surface molecules. Grey lines show the background staining with isotype IgG antibody, whereas black lines show specific staining with the antibodies. Histograms show the results of a representative experiment.

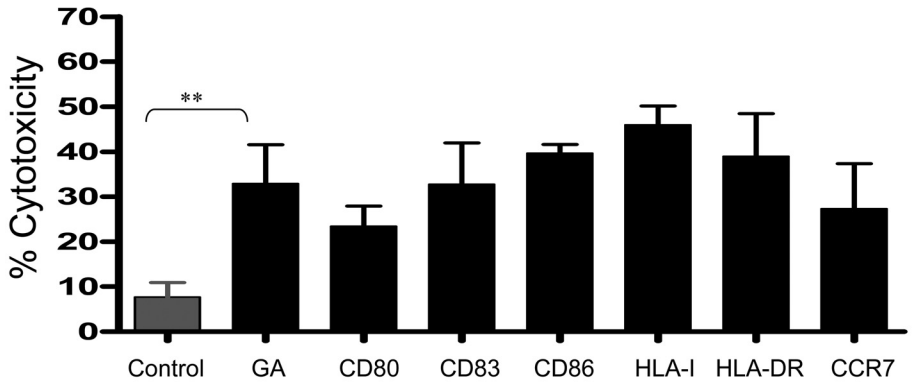

Figure 5. Effects of treatment with co-stimulatory and other molecules on GA-induced enhancement of the cytolysis of mDCs by NK cells. Mature DCs were incubated in the absence (Control) or the presence of 40 μg/ml GA. In other cultures 10 μg/ml anti-CD80, anti-CD83, anti-CD86, anti-HLA-I, anti-HLA-DR and anti-CCR7 were added. Results shown are mean ± SEM of three different experiments. ** = $p < 0.001$, as compared to the control.

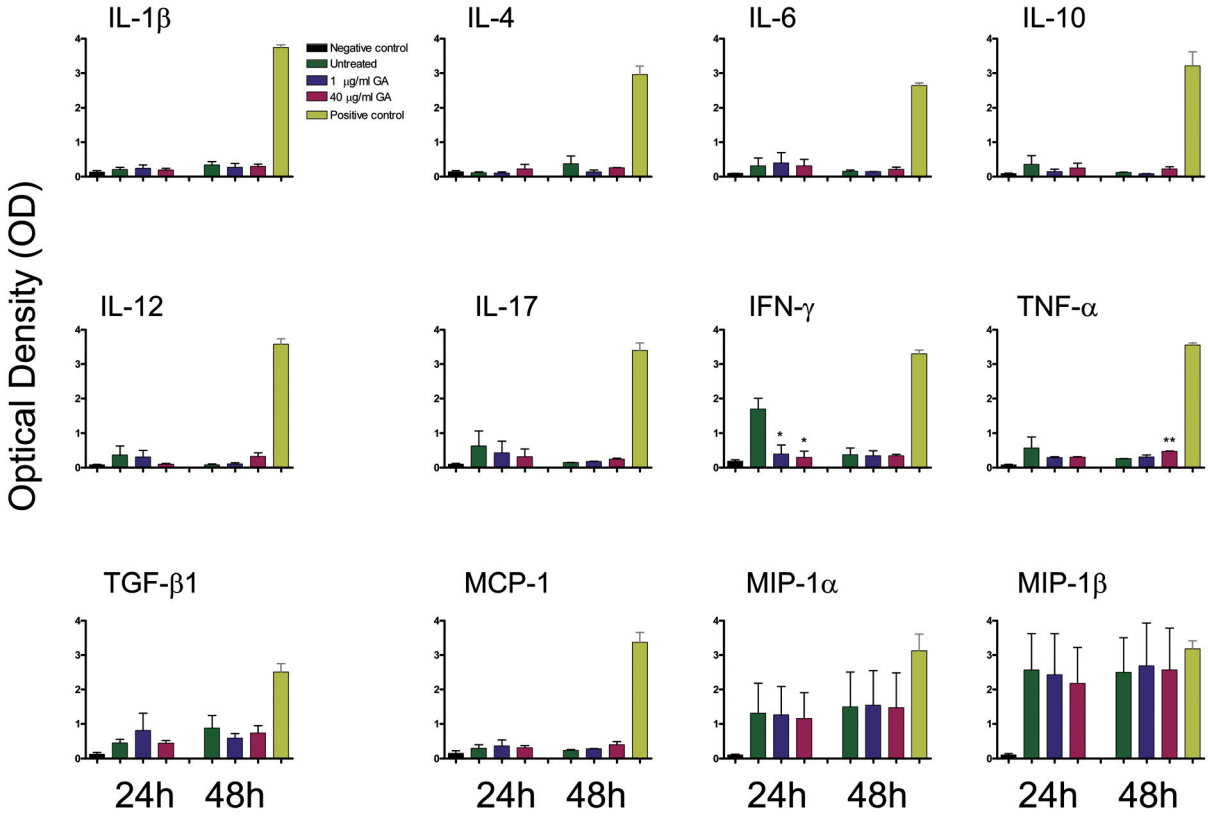

Figure 6. GA inhibits IFN-γ but enhances TNF-α release by activated NK cells. NK cells were incubated either with vehicle (untreated) or with 1 or 40 μg/ml GA for 24 or 48 h. Supernatants were collected and examined for the levels of various cytokines and chemokines. * = $p < 0.05$, and ** = $p < 0.003$. Results shown are mean ± SEM of two different experiments. Positive and negative controls are also included.

of response [1, 4]. In addition, Th2 cells might be recruited into the CNS secreting Th2 cytokines that inhibit type 1 immune response in what is known as a "bystander" mechanism [1]. Recently, several investigators reported that GA affects antigen presenting cells in a way that either impedes their ability to provide antigens or reduces their ability to respond to various stimuli [7–9]. Further, administration of GA into MS patients or EAE mice activates type 2 monocytes which stimulate the anti-inflammatory Th2 cells [8, 9]. Along these lines it was observed that dosing EAE mice with GA resulted in isolating NK cells that show higher cytolysis of immature and mature DCs [16].

In this study we examined the effect of GA on human NK cell lysis of monocyte-derived DCs which are known to support the development of Th1 and not Th2 cells [21], and are implicated in MS/EAE disease [19, 20, 22]. GA enhances human NK cell lysis of immature or mature monocyte-derived DCs, regardless of whether these cells are collected from the same donors as NK cells (autologous) or from different

donors (allogeneic). These results are in line with other findings showing that administration of GA into transgenic Alzheimer's disease mice leads to a significant reduction of $CD11b^+$ microglia cells as compared to untreated mice [23]. Although the mechanism of the disappearance of microglia cells was not examined, it is plausible that NK cells may lyse $CD11b^+$ cells.

Next, we examined the mechanism of action (MOA) of GA. The results indicate that GA affects different molecules in human immature and mature DCs. For example, it is clear that antibodies to NK cell cytotoxicity receptors, i.e. NKp30 (and to a lower extent NKp44) inhibit the enhancing effect of GA on immature but not mature DCs. Our results also indicate that other NK cell receptor(s) important for NK cell mediated cytotoxicity, such as NKG2D is (are) not involved in GA mediated enhancement of activated NK cell lysis of immature or mature DCs. In the literature, it is usually accepted that the MOA of various molecules is investigated either by utilizing antibodies in the cytotoxicity assay or by using flow cytometric analysis to investigate whether these modulatory molecules might affect the percentages of positive cells or their fluorescence intensity. However, we decided to combine the effect of the antibodies in the cytotoxicity assay with the effect of GA on the expression of the NK cytotoxicity receptors. Our results demonstrate that although NKp30 is involved in mediating the effect of GA, this drug does not affect the percentages of cells expressing this molecule or those expressing NKG2D or NKp44, and neither significantly affects the MFI of NKG2D, NKp30 or NKp44 on the surface of NK cells, although the MFI of NKp44 tends to be reduced after incubation with GA. These results indicate that the MOA of GA may be related to modulating the interaction among activated NK cells expressing NK cytotoxicity receptors and iDCs. This could plausibly be related to increasing the affinity of the interaction or to inducing conformational changes in NK cytotoxicity receptors expressed on NK cells, or in their ligands expressed on iDCs.

Similar to NK cytotoxicity receptors, the co-stimulatory molecule CD86 is involved in GA-mediated enhancement of NK cell lysis of iDCs, since addition of anti-CD86 inhibits this lysis. However, GA does not affect the percentages of CD86 expressing iDCs or the MFI of this molecule, suggesting that GA might affect the affinity of binding CD86 to activated NK cells, and as a consequence increases the cytolysis of iDCs. Other molecules such as the co-stimulatory molecules CD80 or CD83, as well as HLA-DR or the chemokine receptor CCR7 might not be involved in GA activity on iDCs, since antibodies to these molecules exert no effect on NK cell lysis of iDCs, although there is a reduction of cytolysis which does not reach statistical significance after incubating the cells with anti-CD80, anti-CD83 or anti-HLA-I. Further, GA does not affect the percentages of iDCs expressing these molecules. An exception is lowering the MFI of HLA-DR on the surface of iDCs after incubation with 40 μg/ml of GA.

In mature DCs, the 40 μg/ml dose of GA reduces the number of cells expressing CD80, CD86 and HLA-DR, suggesting that the MHC class II (DR) molecule might play a role in NK cell lysis of DCs. The other surface molecule that is involved in mature DCs lysis upon stimulation with GA is CD83. In this regard, it should be mentioned that soluble CD83 has been shown to efficiently treat mice with EAE [22]. Whether GA induces the shedding of CD83 facilitating its therapeutic effect remains to be seen. Addition of antibodies to HLA-DR or CD83 does not further reduce the cytolytic activity induced by GA in NK cells against mDCs, since these molecules are already down-regulated by the drug. Interestingly, both concentrations of GA used, i.e. 1 and 40 μg/ml, reduce the number of cells expressing HLA-I, confirming the importance of this molecule in NK cell recognition of target cells. NK cells express inhibitory receptors ensuring that under normal conditions NK cells are inhibited upon ligating self-MHC molecules, which guards against autoimmunity. Hence, Ig inhibitory receptors expressed on NK cells bind MHC class I gene products expressed on target cells, resulting in inhibiting NK cell lysis of those target cells [11]. The results showing that GA reduces the expression of HLA-I on the surface of mDCs may make these cells susceptible to NK cell killing and could partly explain how GA facilitates NK cell lysis of DCs. Finally, the chemokine receptor CCR7, which is important for mature DCs migration into the lymph nodes [24], is not modulated by GA, suggesting that GA does not influence the localization of these cells into the lymph nodes.

Because NK cells secrete multiple cytokines such as IFN-γ as well as various chemokines [25], we examined the array of cytokines and chemokines secreted by these cells. There is a minimal secretion of IL-1β, IL-4, IL-6, IL-10, IL-12, or MCP-1 but low-moderate release of IL-17, TGF-β1, or TNF-α by NK cells left *in vitro* for 24 or 48 h. However, a high release of IFN-γ and the CC chemokines MIP-1α and MIP-1β is observed, confirming previous findings [25]. GA only inhibits the release of IFN-γ 24 h after culture, supporting those findings showing that GA may shift the immune system from Th1 to Th2. Although the Th2 cytokine IL-4 release is not increased after GA

stimulation of NK cells, this should not be surprising considering that NK cells may not secrete IL-4.

This is the first demonstration that a drug is capable of activating NK cells to lyse monocyte-derived DCs, a concept that may have vital implications. First, it appears that in addition to inhibiting IFN-γ release and switching the system toward Th2, GA may shut down the Th1 axis pathway, perhaps by ridding the system of monocyte-derived DCs that activate Th1 cells. The fact that NK cells exposed to GA kill both immature and mature DCs ensures that no antigen presentation would be available to Th1 cells. Also, this ensures that during chronic infection, where microbes survive the initial attack of the immune system, any stimulation of Th1 cells by monocyte-derived mDCs would be monitored and controlled by NK cells. The results also indicate that even when allogeneic DCs are administered inside the body (for example during allogeneic bone marrow transplantation), NK cells may kill these cells, preventing them from processing and presenting antigens to autoreactive T cells. This new mechanism may complement the already known MOA of GA, which activates type 2 monocytes that support the activation of Th2 cells as well Treg cells [8, 9]. It is assumed that the activity of GA is directed toward enhancing NK cell lysis of monocyte-derived DCs which stimulate Th1 but not toward plasmacytoid DCs which stimulate Th2 cells, although this has not been examined.

The ability of GA to enhance NK cell lysis of DCs may not be exclusive to this drug, as other findings showed that the monoclonal antibody, Daclizumab, enhances the lytic activity of $CD56^+$ NK cells, corroborated with the ability of this antibody to reduce brain inflammation in MS patients [26], albeit the effect of NK cells on DCs was not examined in this study. Beside Daclizumab, anti-CD3, anti-CD4 (cM-T412), anti-CD52 (Campath-1H, or Alemtuzumab), or anti-CD20 (Rituximab) used for treatment of MS patients, have a common effect of depleting autoreactive T cells and/or antigen presenting cells such as dendritic cells [27, 28]. The possibility that these antibodies might activate NK cells to lyse DCs is intriguing.

Our results may also explain how GA, alone or in combination with other immunosuppressive drugs, prolongs skin graft survival and inhibits graft versus host (GvH) disease [2]. In this disease, DCs of the host provide antigenic stimulation to T cells of the donor which consequently attack host cells. The ability of GA to stimulate NK cells to lyse allogeneic immature and mature DCs provides new knowledge to the basis of utilizing GA for the treatment of allograft rejection.

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