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Effects of Natural Suppressor Cells on Production of Th1 and Th2 Cytokines and Proliferation of Polarized T Cells

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Natural suppressor cells from the bone marrow of intact animals and mice with Th1- and Th2-dependent immune response largely suppressed the production of Th1 (IFN-γ, IL-2) and Th2 cytokines (IL-4). Antiproliferative activity of natural suppressor cells from intact and immunized animals was mediated by NO. Proliferation of Th1 cells was suppressed greater than that of Th2 cells. This selective effect of natural suppressor cells on the proliferation of target lymphocytes was due to higher stimulatory potential of Th1 cells (in comparison with Th2) towards natural suppressor cells.

Key Words: *natural suppressor cells; interferon-γ; interleukin-2; interleukin-4*

The key role in the regulation of immunocompetent cell is played by T helpers which under the effect of an antigenic stimulus irreversably differentiate into two subclasses (Th1 and Th2), differing by the profile of cytokines produced by them [3,11]. Cells regulating the immune response include hemopoietic cells of different maturity, which nonspecifically suppress lymphocyte proliferation: natural suppressor cells (NSC) [4,7,12]. Activity of NSC increases significantly during tumor growth [2,15], pregnancy [7], development of graft-versus-host reaction [5], cyclophosphamide treatment [12], nematode (*Brugia malayi*) infection [9], and after injection of superantigen [14]. We previously showed that activity of NSC increases during the development of Th1- and Th2-dependent immune response [1].

The data on the effects of NSC on cytokine production by T cells are scanty. The majority of authors studied the effects of NSC on the production of IL-2, but the results are contradictory [6-8,10,13]. It was reported than NSC disordered IL-4 utilization, but there are no data about their effects on the production of this cytokine [6,7].

We studied the effects of NSC from the bone marrow of intact animals and mice with Th1- and Th2-dependent immune response on the production of Th1 and Th2 cytokines and on the proliferation of lymphocytes polarized by types 1 and 2.

MATERIALS AND METHODS

Experiments were carried out on 8-12-week-old BALB/cY mice (n=105) of both sexes from Breeding Center of Institute of Pharmacology (1st category certified animals). The mice were kept in incomplete barrier system with free access to sterile granulated fodder and boiled drinking water acidified with hydrochloric acid to pH 4.0-4.5.

Th1 and Th2 cells were isolated as described previously [1]. The mice were immunized 3 times with BCG (N. F. Gamaleya Institute of Epidemiology and Microbiology) or ovalbumin (OVA; Sig-

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ma). BCG (100 μg) was injected subcutaneously into the tail base in 0.1 ml incomplete Freund's adjuvant (Difco). OVA (100 μg) was injected under the skin on the thigh (0.1 ml) with aluminum hydroxide (5 mg/mouse; Sigma) as an adjuvant. Controls received 0.1 ml isotonic NaCl solution. Repeated immunizations were carried out at 14-day intervals; the material was collected on day 7 after the last injection. Polarization type was determined by the cytokine profile.

In order to prepare cell suspensions, the mice were sacrificed by cervical dislocation, bone marrow cells were obtained by perfusion of the femoral and tibial bones, splenocytes were isolated after homogenization of the spleens. Cell suspensions were filtered through 4 layers of capron, washed, resuspended in culture medium, and their viability was evaluated.

In order to isolate NSC, adherent cells were removed from myelokaryocyte suspension after culturing for 15-17 h in plastic Petri dishes (Costar), after which cells with density below 1.077 g/liter were isolated by fractionation in Histopaque-1077 (ρ=1.077 g/ml, Sigma-Aldrich). The cells were cultured at 5% CO_2 and absolute humidity in RPMI-1640 (Sigma) with 10% ECS (ICN), 20 mM HEPES (Sigma), 0.05 mM 2-mercaptoethanol (Sigma), 50 μg/ml gentamicin (Flow Lab.), and 2 mM L-glutamine (Flow Lab.).

For evaluation of the effect of NSC on cytokine production by splenic T cells, effector cells (2×10^5/well) were cultured for 60-64 h (IFN-γ and IL-4 assay) or 36 h (IL-2 assay) in 96-well plates with target cells (2×10^5/well) and 4 μg/ml Con A (Sigma), after which the supernatant was collected and cytokines were assayed. Control supernatants were collected from cultures containing no effector cells. The content of IL-4 was measured by enzyme immunoassay using a commercial test system (Amersham Biosciences), IFN-γ and IL-2 in biotests by the capacity of lymphocyte supernatant to induce NO production by myelokaryocytes of intact syngeneic animals or stimulate the proliferation of IL-2-dependent splenocytes. Supernatant of T cells was added to 96-well plates (Costar) with fresh-isolated myelokaryocytes ($2\text{-}3\times10^5$/well), cultured for 48 h, supernatant was collected, and nitrites were measured using Griess reagent. Changes in the production of IFN-γ were evaluated by the difference in nitrite content in supernatants before the biotest (zero level) and the resultant content in the biotest. IL-2-dependent lymphocytes were obtained by culturing splenocytes (4×10^6/ml) with Con A (4 μg/ml) for 36 h. After removal of the mitogen splenocytes were put into 96-well plates (5×10^4 cell/well) with supernatant, cultured for 48 h, and their proliferation was evaluated. For evaluation of cell proliferation, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Serva) was added 4 h before the end of culturing to a concentration of 200 μg/ml. After removal of the supernatant the precipitate was dissolved in DMSO (Tatkhimfarm-

TABLE 1. Immunosuppressive (% of Suppression) and NO-Producing (Nitrite Concentration, μM) Activities of Bone Marrow Cells Towards Lymphocytes of Intact and OVA- or BCG-Immunized Animals ($X\pm m$)

NSC and lymphocyte sources		Immunosuppressive activity		NO-producing activity	
		effector/target			
		0.5/1.0	1/1	0.5/1.0	1/1
Intact mice	intact (control)	50.7±1.1	67.7±3.4	20.9±0.8	27.1±0.7
	BCG (experiment)	64.8±3.6*	84.2±2.3*	26.0±0.8*	34.6±2.1*
	intact (control)	49.1±7.1	68.5±5.8	16.9±1.0	34.2±0.4
	OVA (experiment)	16.8±5.3*	43.8±0.3*	6.6±2.0*	22.6±2.1*
BCG	intact (control)	54.3±1.2	75.2±3.1	26.7±0.5	35.5±1.8
	BCG (experiment)	73.8±3.7*	91.3±2.4*	32.8±0.7*	43.4±0.9*
	intact (control)	80.4±3.6	92.0±2.6	37.4±3.5	44.9±1.6
	OVA (experiment)	48.5±5.5*	74.4±4.5*	20.9±4.1*	29.6±3.3*
OVA	intact (control)	69.7±5.9	83.1±2.3	31.5±2.1	38.0±3.8
	BCG (experiment)	92.5±2.7*	97.1±0.9*	52.9±2.3*	63.6±2.8*
	intact (control)	54.4±3.6	72.2±3.2	20.1±0.7	40.2±1.8
	OVA (experiment)	24.1±1.1*	45.7±6.0*	13.9±1.0*	30.0±0.5*

Note. *$p<0.05$ compared to the control.

preparaty) and absorption of solutions was measured at λ=550 nm. The index of IL-2-dependent splenocyte stimulation (SI) was evaluated by the formula: SI=OD1-OD2, where OD1 is optic density in the wells with IL-2-dependent splenocytes with supernatant and OD2 optic density in wells with IL-2-dependent splenocytes without supernatant.

Activity of NSC was evaluated by suppression of proliferation of Con A-stimulated splenic syngeneic lymphocytes. NSC were cultured for 60-64 h in 96-well plates with target cells (2×10^5/well) in different ratios with 4 μg/ml Con A. The label (^{3}H-thymidine, 0.5 μCi/well) was added 16 h before the end of culturing; label incorporation was evaluated on a β-scintillator. Suppressor activity (%) was estimated by the formula: 100×(1-TE/T), where TE is count cpm in wells with effector cells+target cells and T is count cpm in wells with target cells.

The data were processed using Statistics 6.0 software and Student's *t* test.

RESULTS

NSC significantly inhibited production of IFN-γ (Fig. 1, *a*): this parameter decreased to 55.3% in the presence of intact mouse NSC, to 54.6% in the presence of NSC from animals immunized with BCG, and to 58.9% of control (no NSC) in the presence of NSC from mice immunized with OVA. The production of IL-2 by T cells also decreased in the presence of NSC (Fig. 1, *b*) to 39.9% (intact mouse NSC), 20% (NSC from BCG-immunized animals), and 38.9% of control (NSC from OVA-immunized animals). NSC inhibited the secretion of IL-4 by T cells of intact animals (Fig. 1, *c*). The level of IL-4 production in the presence of NSC decreased to 42.2% (intact mouse cells), 51.5% (BCG-immunized mouse cells), and 33.4% (OVA-immunized mouse cells) of control level.

The next step of our study was to compare NSC effects on the proliferation of polarized and naive

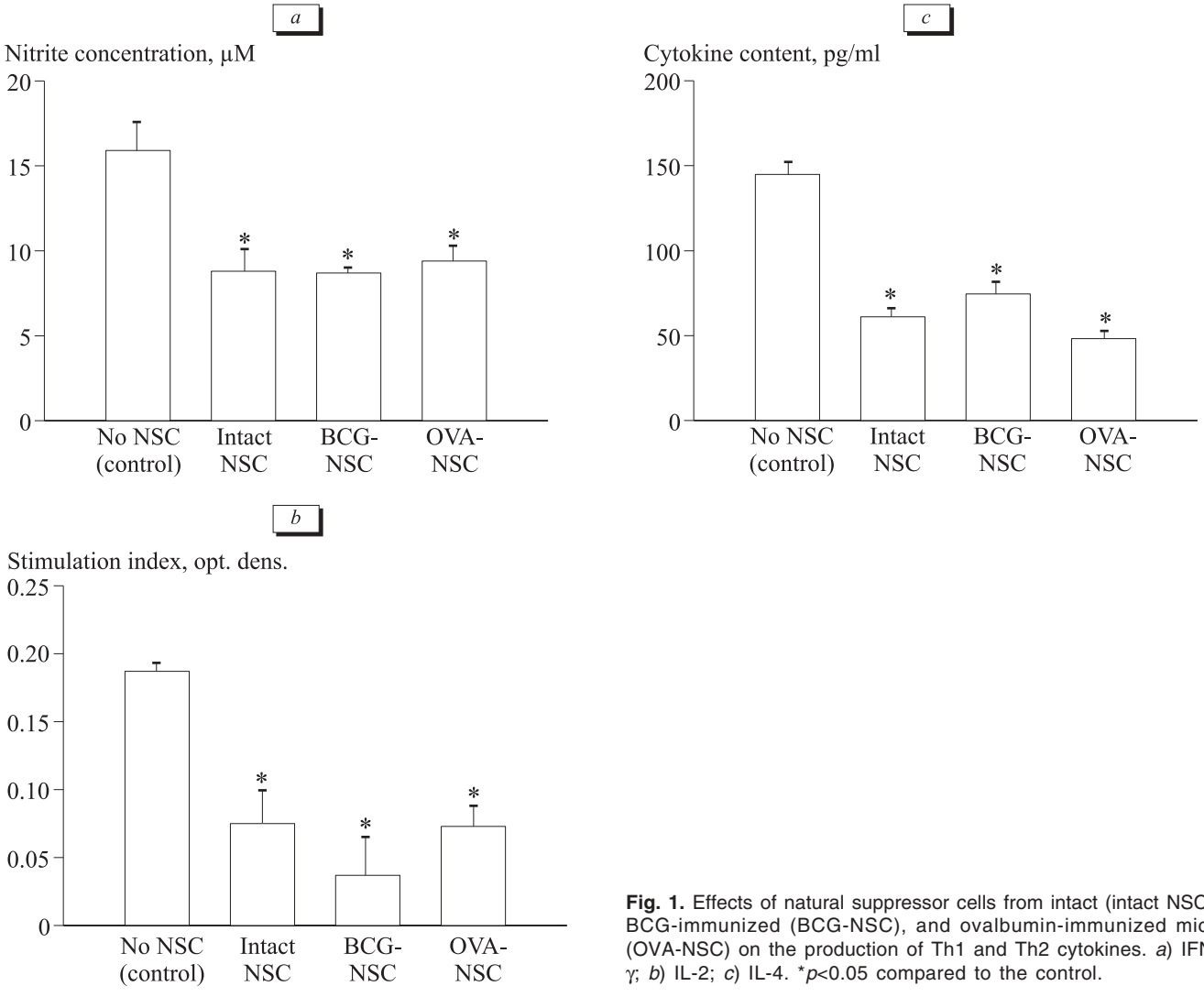

Fig. 1. Effects of natural suppressor cells from intact (intact NSC), BCG-immunized (BCG-NSC), and ovalbumin-immunized mice (OVA-NSC) on the production of Th1 and Th2 cytokines. *a*) IFN-γ; *b*) IL-2; *c*) IL-4. *$p<0.05$ compared to the control.

lymphocytes (Table 1). NSC from intact and immunized animals more intensely suppressed the proliferation of Th1 lymphocytes than of naive T cells, but were less active towards Th2 lymphocytes in comparison with naive cells. Increase of antiproliferative activity (towards Th1) was paralleled by a higher level of NO production by myelokaryocytes; lower antiproliferative activity (towards Th2) was associated with lower production of NO.

Hence, irrespective of NSC source (intact animals or animals with developing immune response) we observed no selective effect of NSC on cytokines: the production of Th1 and Th2 cytokines was suppressed. The effects of intact NSC and NSC from immune animals on lymphocyte proliferation were selective: the cells exhibited a higher antiproliferative activity towards Th1 *vs.* Th2 cells. The NO-dependent mechanism underlies this effect. It seems that the selective effect of NSC on polarized type 1 T cells is not due to the predominant suppression of Th1 cytokines, but to predominant activation of NSC by these cytokines. Lymphocytes from animals with Th1-dependent immune response, producing more IFN-γ than lymphocytes of intact or OVA-immunized mice, more intensely stimulate the production of NO; in other words, selective effect on the proliferation of target lymphocytes is due to higher capacity of Th1 cells (in comparison with Th2 ones) to stimulate the production of NO by NSC.

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