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γδT Cells Positively Regulate Contact Sensitivity (CS) Reaction via Modulation of INF-γ, IL-12 and TNF-α Production*

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The γδ T cells were identified as positive as well as negative regulators of immune responses. They take part in pathogen clearance, modulation of innate and adaptive immunity as well as in healing and tissue maintenance. The course of many pathological conditions such as collagen induced arthritis (CIA), experimental autoimmune encephalomyelitis (EAE) and airway hyperresponsiveness is positively regulated by γδ T cells. It was shown previously that contact sensitivity (CS), an example of antigen-specific cell-mediated immune response, is also positively regulated by γδT cells. The current work confirmed the regulatory function of γδT cells in CS response as their depletion with anti-TCRδ monoclonal antibody and complement significantly decreased adoptive transfer of the CS reaction. *In vitro* study showed that removal of γδT cells with magnetic beads significantly decreased the production of the proinflammatory cytokines IFN-γ, IL-12 and TNF-α. Reconstitution of γδT-depleted cells with γδT-enriched cells restored cytokine production, proving the reversibility of the investigated process. In summary, γδT cells positively regulate the CS reaction via modulation of proinflammatory cytokine production.

Key words: Contact sensitivity, gamma-delta T cells, contrasuppression, adoptive transfer, cytokines.

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The contact sensitivity (CS) reaction is an antigen-specific cell-mediated immune response, which is a classical example of delayed-type hypersensitivity (DTH). Antigen-specific CD4+ Th1 effector cells are induced as a result of skin exposition to haptens that are low molecular weight compounds (MAJEWSKA *et al.* 2009). The typical contact sensitivity response in humans is allergic contact dermatitis. It is notable that allergic contact dermatitis resulting from exposure to chemicals in the workplace constitutes about 30% of all occupational diseases (DIEPGEN & WEISSHAAR 2007). This type of response may occur due to long-term exposure of the skin to low molecular weight substances, including heavy metals (e.g. chromium, nickel, cobalt), latex, turpentine, fragrances and preservatives in cosmetics, epoxy resins and their hardeners, as well as some drugs applied in ointments (e.g. Neomycin).

The contact sensitivity reaction consists of two subsequent stages known as induction and elicitation. Antigen-specific CD4+ Th1 cells are induced after topical application of a hapten such as TNP-Cl. Repeated exposure to such compounds elicits migration of previously induced TNP-specific T cells to the site of elicitation, leading to a local inflammatory response in the skin. Clinical manifestations of this reaction involve edema, erythema as well as oozed papules, pustules and itching.

Although the mechanism of CS is well known, its regulation is still elusive. It was previously shown that the population of innate-like lymphocytes that express γδTCR is involved in positive regulation of the CS reaction in mice (PTAK & ASKENASE 1992). These positively acting antigen-non-specific γδT cells are required to assist αβT cells in the successful adoptive transfer of the CS response (ASKENASE *et al.* 1995). Finally it

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was shown that CS-assisting γδT cells belong to a rare population of lymphocytes that express Vγ5 and Vδ4 regions of TCR (PTAK *et al.* 1996).

On the other hand experiments employing TCRα-/- mice showed that γδT cells can negatively regulate the CS response in mice. Described suppression was antigen-specific but not restricted by MHC (SZCZEPANIK *et al.* 1996). These suppressor cells belong to the population of $CD28^{+}CD40\text{-}Ligand^{+}$ Fas^{+} $Fc_{\gamma}R^{+}$ $NK1.1^{-}$ γδT lymphocytes that inhibit the CS reaction via IL-4 (SZCZEPANIK *et al.* 1999).

The immunoregulatory function of γδT cells was also recognized in other experimental models. It was shown that γδT cells support inflammatory response in graft-vs-host disease (TSUJI *et al.* 1996) and autoimmune diseases such as systemic lupus erythematous, rheumatoid arthritis and experimental autoimmune encephalomyelitis (EAE) (HAYDAY & TIGELAAR 2003; PONOMAREV *et al.* 2004). γδT cells enhance conversion of naive αβT cells into proinflammatory CD4+ Th1 lymphocytes by production of IFN-gamma (YIN *et al.* 2000). In a model of EAE, induction of encephalitogenic αβT cells is also enhanced by IL-12 produced by antigen presenting cells (APC) supplemented by γδT cells (ODYNIEC *et al.* 2004). Furthermore, IL-23 activated γδT cells shield αβT effector cells from the suppressive activity of Treg cells as well they inhibit formation of inducible Treg cells (PETERMANN *et al*. 2010).

The signals from γδT cells that enhance or suppress the immune response were not described precisely. It was shown that blood derived γδT cells that express the Vγ1 or Vγ4 subunits are programmed to produce IFN-γ and IL-17A, respectively. Both cytokines are known to aggravate autoimmune conditions.

The current work focuses on further characterization of CS-assisting γδT cells and the mechanism of their protective action in the CS reaction.

Material and Methods

Mice

Specific pathogen free (SPF) male CBA/J mice from the breeding unit of the Department of Medical Biology, Jagiellonian University, College of Medicine were used.

Mice were maintained under specific pathogen-free conditions, and used at 10-12 weeks of age in groups of 10. All experiments were conducted according to the guidelines of the Jagiellonian University College of Medicine (No of approval 70/2010).

Reagents

2-merkaptoethanol (Sigma, St. Louis, MO, USA); amino acids (NEAA; Gibco, Grand Island, NY, USA); anty-TCRδ (UC7-13D5) from Dr J. Blueston (University of California, San Fransisco, CA., USA); rabbit complement (RC; Pel-Freeze Biologicals, Brown Deer, WI, USA); buffer HEPES (Gibco, Grand Island, NY, USA); fetal calf serum (FCS) (PAA Laboratories, Pasching, Austria); IFN-γ, TNF-α, IL-17A BD OptEIA Set (BD Biosecienes, San Diego, CA., USA); L-glutamine (Sigma, St. Louis, MO, USA); penicillin and streptomycin (Gibco, Grand Island, NY, USA); RPMI 1640 (Gibco, Grand Island, NY, USA); TNP-Cl, picryl chloride (Chemica Alta, Edmonton, Canada); γδT cell separation kit (Miltenyi BioTek GmbH, Auburn, CA, USA); mouse immunoglobulins (Ig) were prepared from CBA/J mouse sera and conjugated with TNP hapten (Little and Eisen, 1966) . A single preparation with the level of substitution of 40 TNP per Ig molecule (TNP_{40}-Ig) was used for *in vitro* study.

Active immunization and adoptive cell transfer of CS

Donors of immune cells were actively contact sensitized by topical application of 0.15 ml of 5% TNP-PCl in a 1:3 acetone: ethanol mixture, to the shaved abdomen, chest and hind feet on day 0. On day 4, lymph nodes were harvested and a single cell suspension was prepared. Then, a mixture of 7×10^{7} immune inguinal and axillary lymph node cells or an equivalent number of γδT cell depleted lymph node cells were transferred adoptively intravenously (*i.v.*) into naive syngeneic recipients. Immediately after the transfers, recipients were challenged on each ear with 10 μl 0.4% TNP-PCl in olive oil:acetone, 1:1. Subsequent increase in ear swelling was determined 24 h later using a micrometer (Mitutoyo, Paramus, NJ), and expressed in μm ± SE. Each experiment consisted of a group of non-immune mice that were only challenged on the ears with 0.4% PCl, and their background ear swelling (± 20 μm at 24 h), was subtracted from the responses of the experimental groups, to yield the net ear swelling responses that are shown in the figure.

Complement-mediated depletion of γδT cells

To confirm the regulatory role of γδT cells in adoptive cell transfer of CS reaction, γδT cells were removed from the lymph nodes isolated from TNP-PCl actively immunized mice. Lymph node cells isolated from mice immunized with TNP-Cl, were incubated in PBS with anti-TCRδ mAb, clone UC7-13D5 (1 μg Ab/10^{6} cells) or only in

PBS on ice for 60 min. Then, cells were washed with PBS and incubated at 37°C with a predetermined dilution (1:25) of RC for 45 min. Next, the cells were washed, resuspended in an adequate volume of PBS and *i.v.* transferred into naïve recipients (adoptive transfer), which were subsequently ear challenged and tested for CS response.

Immuno-magnetic bead γδ T cell fractionation

Lymph node cells from TNP-Cl immunized mice were isolated and a single cell suspension was prepared under aseptic conditions. The cells were washed in ice cold PBS and counted. After centrifugation at 300xg for 10 min, the cell pellet was resuspended in 450 μl of buffer for magnetic cell sorting per 10^8 total cells and mixed with 50 μl of a non-T cell depletion cocktail (magnetically labeled anti-CD45R and anti-CD11b mAbs) and 50 μl of biotinylated anti-pan-TCRγδ mAb. After 15 min incubation at 4°C, the cells were washed with buffer. The cell pellet was resuspended in 500 μl of buffer for magnetic cell sorting containing $1.25x10^8$ total cells. The cell mixture was applied to the column and separated in a magnetic field. The collected effluent contained the unlabeled pre-enriched T cell fraction. The collected cell population was washed by centrifugation at 300xg for 10 min and then the cell pellet was resuspended in 450 μl of buffer and mixed with anti-biotin microbeads. The cells and anti-biotin microbeads were incubated for 15 min at 4°C. Then, the cell suspension was applied on a column rinsed with 2 ml of the buffer. The cells that passed through the column were collected and the column was washed twice. The γδT cells present in the column were flushed out and collected. The cells were then cultured *in vitro*.

In Vitro Culture

$3x10^6$ of whole lymph node cell or an equivalent of γδT cell depleted lymph node cells were cultured in a 24-well plate in 1 ml of RPMI 1640 medium supplemented with 5% FCS in the presence of 100 μg/ml TNP_{40}-Ig as the antigen. The reconstitution control group containing γδT cell depleted lymph node cells supplemented with γδT lymphocytes was cultured with TNP-Ig antigen. After 48 hr culture the supernatants were collected and cytokine production was measured with the use of the BD OptEIA Set (BD Bioseciences, Sam Diego, CA., USA).

Statistical significance was measured using one-way ANOVA. The results were considered as significant when $P<0.05$.

Results

Previous studies reported that γδT cells have immunoregulatory activity in CS response and other experimental models. To determine the role of γδT cells in CS, adoptive cell transfer of γδT cell depleted TNP-specific CS-effector cells was performed. Data presented in Figure 1 show that depletion of γδT cells before *i.v.* cell transfer significantly decreased adoptive transfer of CS when compared to positive control (Group B vs A).

To determine the mechanism of γδT cell-mediated immunoregulation of CS, cytokine production was evaluated *in vitro*. Using the MACS

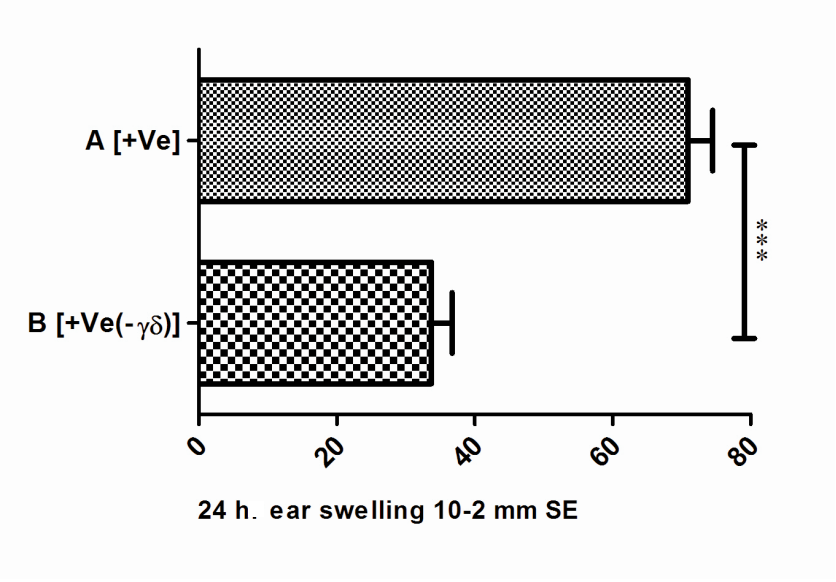

Fig. 1. Depletion of γδT cells before *i.v.* cell transfer decreased adoptive transfer of CS. Group A [+Ve] – transfer of whole CS-effector cells; Group B [+Ve(-γδ)] – γδT cells depleted CS-effector cells; Results presented as mean values in μm± SE, n=8; ***P<0.001.

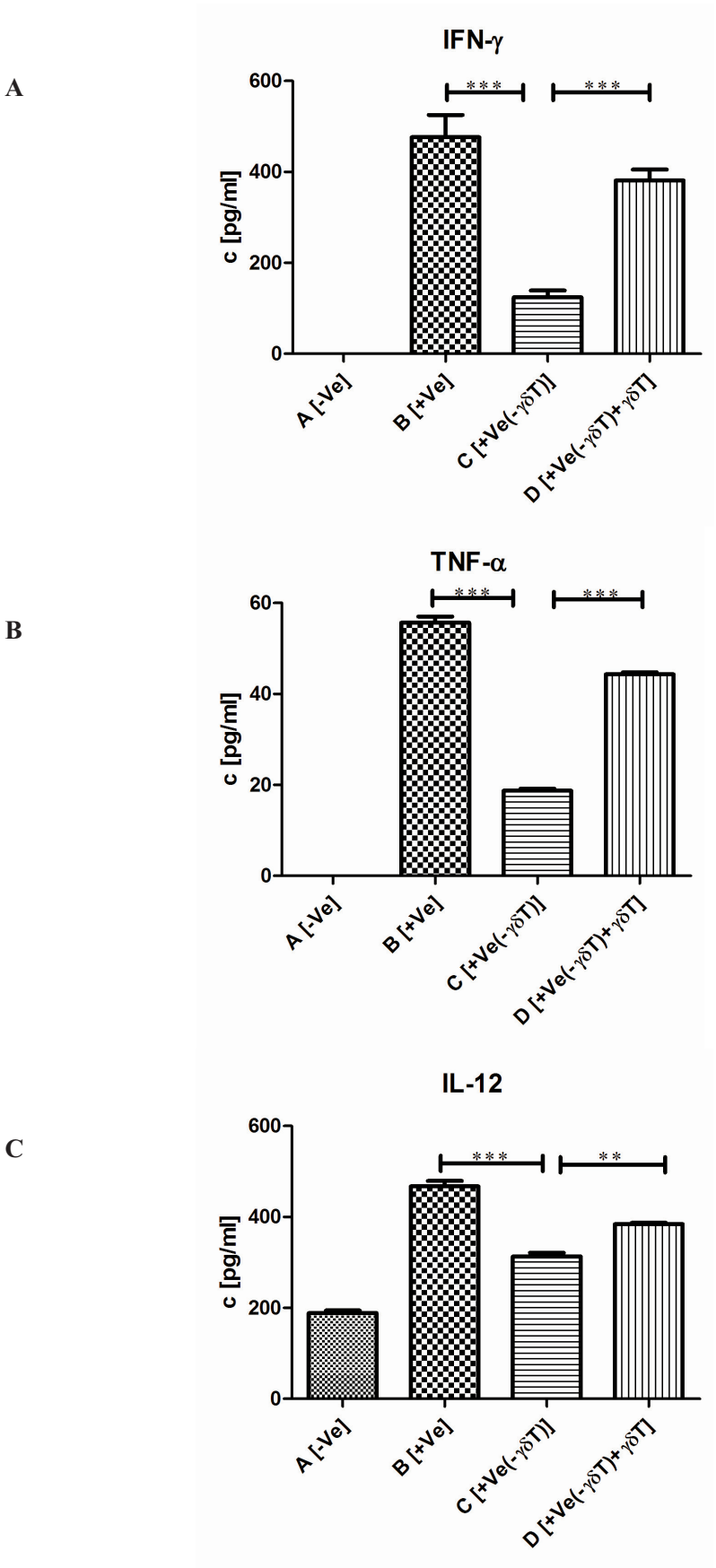

Fig. 2. IFN-γ, IL-12, TNF-α concentration in culture supernatants. Group A [-Ve] - negative control; B [+Ve] – whole 4 day TNP-Cl immune cells; C [+Ve(-γδ)] - γδT cell depleted 4 day TNP-Cl immune cells; D [+Ve(-γδ)- γδT] – γδT cell depleted 4day TNP-Cl immune cell reconstituted with γδT cells; Results presented as means value in pg/ml ± SE, n=4; **P<0.01, ***p.001.

sorting system, lymph node cells from TNP-Cl immune mice were separated into γδT cell-depleted and γδT cell-remaining populations. Then whole or γδT cell-depleted cells were cultured as described in Materials and Methods (Groups B and C, respectively). Additionally, one group of γδT cell-depleted cells was reconstituted with previously isolated γδT cells (Group D) in order to check the reversibility of γδT cell depletion. Lymph node cells from naïve mice were used as a negative control (Group A). Cytokine production was evaluated in culture supernatants. Data presented in Figure 2 show that γδT cells play an important role in the production of pro-inflammatory cytokines as γδT depletion significantly reduced IFN-γ (Fig. 2A; Group C vs B), TNF-α (Fig. 2B; Group C vs B) and IL-12 (Fig. 2C; Group C vs B) production. The production of IL-17A and IL-6 was below detection level (data not shown). Reconstitution of γδT-depleted cells with γδT-enriched cells restored cytokine production (Fig. 2A-C; Group D vs C).

Discussion

γδT cells are involved in many biological processes such as pathogen clearance, modulation of innate and adaptive immunity as well as healing and tissue maintenance. The aforementioned functions are maintained by γδT cells that have the ability to secrete cytokines such as IFN-γ and IL-17A, produce factors that promote tissue healing and regeneration as well as the capacity to kill infected or transformed cells (BONNEVILLE *et al.* 2010).

The immunomostimulatory role of γδT cells has been previously shown in animal models of CIA (ROARK *et al.* 2007), EAE (PONOMAREV *et al.* 2004) and airway hyperresponsiveness (COOK *et al.* 2008). γδT cells were also identified as a cell population that assists αβT CS-effector cells (PTAK *et al.* 1996; SZCZEPANIK *et al.* 1998). The mechanism of γδT assistance was not known until now.

It was previously shown that γδT cells can secrete high amount of pro-inflammatory INF-γ after PHA activation in a Th1-promoting environment (YIN *et al.* 2000). Furthermore, secreted IFN-γ provides an environment favoring the development of antigen specific αβT effector cells directly or indirectly stimulating IL-12 production by APC (DEVILDER *et al.* 2006). Elicitation of the CS reaction is also dependent on activity of γδT which secrete cytokines that optimize the function of αβT CS-effector cells (YOKOZEKI *et al.* 2001).

Our results indicate down-regulation of the CS reaction after adoptive transfer of lymph node cells from TNP-Cl immunized mice depleted of γδT cells (Figure 1), and correspond to previous observations showing that γδT cells support development of the CS reaction (ASKENASE *et al.* 1995). In order to clarify the role of γδT cells in CS we measured the production of cytokines in culture supernatants. Data presented in Fig. 2C show that depletion of γδT cells significantly inhibits IL-12 production. This data may suggest that γδT cells deliver stimulatory signals that are required for IL-12 production by APC. Then, released IL-12 supports induction of Th1 CS-effector cells. This is in line with other reports showing that γδT cell activated APC to secrete IL-12 that is essential for activation of T-effector cells in an animal model of multiple sclerosis (EAE) (ODYNIEC *et al.* 2004). On the other hand it was previously shown that IL-12 can also exert protective signals and act as a factor that strengthens the resistance of CS-effector T cells to suppressive activity of Treg cells (SZCZEPANIK *et al.* 1998; SZCZEPANIK and ASKENASE 2000; PTAK *et al.* 2000; KAWAMOTO *et al.* 2000; SZCZEPANIK *et al.* 2000).

Our *in vitro* experiments also showed that depletion of γδT cells significantly diminished IFN-γ and TNF-α production (Figures 2A and 2B respectively). Both cytokines are involved in the effector phase of CS. It was previously shown that after elicitation of the CS response, recruited αβTCR effector cells specific for Ag/MHC complexes on APC are activated to produce pro-inflammatory cytokines such as IFN-γ (VAN LOVEREN *et al.* 1984). Locally generated IFN-γ leads to recruitment of circulating leukocytes including macrophages that secrete TNF-α after stimulation with IFN-γ (KIMBER & DEARMAN 2002). Thus, our data suggest that γδT cells are also involved in the effector phase of CS supporting IFN-γ production by CS-effector T cells and subsequent TNF-α release by macrophages to strengthen CS-elicitation (PONOMAREV *et al.* 2004). It is also possible that γδT cells are able to stimulate activity of αβT CS-effector cells indirectly via IFN-γ dependent activation of APC (KAWAMOTO *et al.* 2000; DEVILDER *et al.* 2006).

In summary, the current work confirms a supporting role of γδT cells in the CS response and shows that this regulatory effect is mediated by proinflammatory cytokines.

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