

Human CD4⁻ CD8⁻ Invariant Natural Killer T Cells Promote IgG Secretion from B Cells Stimulated by Cross-Linking of Their Antigen Receptors

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Abstract

Immunoglobulin (Ig) M production can be induced by the interaction of thymus-independent type-2 (TI-2) antigen (Ag) with B cell Ag receptors (BCRs) without the involvement of conventional T cells; for IgG production through the same process, however, a second signal is required. Previous studies have reported that invariant natural killer T (iNKT) cells may be responsible for the second signal involved in IgG production. In the present study, we addressed whether human iNKT cells could participate in the production of Ig against TI-2 Ag *in vitro*. Two major distinct subsets of human iNKT cells, CD4⁺ CD8 β ⁻ (CD4) and CD4⁻ CD8 β ⁻ [double negative (DN)] cells, were generated from peripheral blood monocytes from a healthy volunteer. BCR engagement, triggered by anti-IgM antibody stimulation, examined here as a model of BCR engagement triggered by TI-2 Ag, induced abundant IgM production by B cells. Both CD4 and DN iNKT cells reduced IgM production and conversely enhanced IgG production in a dose-dependent manner. In addition, IgG production by CD19⁺CD27⁻ (naïve) and CD19⁺CD27⁺ (memory) B cells was predominantly promoted by DN

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iNKT cells rather than CD4 iNKT cells; nevertheless, IgM production by both B cell subsets was similarly reduced by either subset of iNKT cells. These results suggest that the DN iNKT subsets may preferentially promote Ig class switching by B cells upon stimulation with TI-2 Ag.

Keywords

Invariant Natural Killer T Cells, TI-2 Antigen, B Cells, IgM, IgG

1. Introduction

The main causative bacteria of invasive infection, including *Streptococcus pneumoniae* and *Haemophilus influenzae*, possess thick polysaccharide capsules which confer the ability to resist phagocytosis by polymorphonuclear leukocytes [1]. Immunoglobulins specific for these polysaccharide capsules enhances opsonophagocytic killing (OPK) activity, which plays an important role in host protection against infections caused by these encapsulated bacteria [2] [3]. Recently, we reported that immunization with pneumococcal polysaccharide vaccine led to an increase in the serum level of serotype 3-specific IgG3, which facilitates survival after pneumococcal infection in mice [4]. Immunization with pneumococcal polysaccharide vaccine also generates polysaccharide-specific IgG response in humans [5] [6].

Polysaccharide capsule, a thymus-independent type 2 (TI-2) antigen (Ag), has highly repetitive structures with simultaneous cross-linking of B cell receptors (BCRs) and induces B cell proliferation and IgM production [7]. The antibody response induced by TI-2 Ag is smaller than that induced by thymus-dependent (TD) Ag, and consists largely of the production of low-affinity IgM by B cells without the conventionally necessary T cell involvement. In addition, however, IFN- γ induces T cell-independent IgG production in response to TI-2 Ag [8], as this cytokine triggers the secondary stimulatory signals for T cell-independent B cell activation and isotype switching to produce IgG [9] [10].

Human invariant natural killer T (iNKT) cells express only two $\alpha\beta$ T cell Ag receptors, namely, $V\alpha 24$ - $J\alpha 18$ and $V\beta 11$, and have been identified as a unique lymphocyte population playing a critical role in both innate and adaptive immune responses [11] [12]. Although $V\alpha 24^+V\beta 11^+$ iNKT cells are present only in very small proportions (<0.01% - 1%) in human blood [13], these cells recognize glycolipids from bacteria and/or self in context with CD1d, a nonpolymorphic MHC class I-like molecule, which leads to the production of large quantities of cytokines such as IFN- γ , IL-4, IL-10 and IL-17A [14]. Human $V\alpha 24^+$ iNKT cells comprise two distinct major subpopulations, one expressing $CD4^+CD8\beta^-$ (CD4) and the other $CD4^-CD8^-$ [double negative (DN)] [13]. These two subsets of iNKT cells differ in terms of the cytokines they produce to regulate various immune responses [15]. Mice lacking iNKT cells exhibit defective IgG response to pneumococcal polysaccharide Ags, intact response to TD Ags [16] and impaired host defense against pneumococcal infection [17]. These previous observations suggest that iNKT cells may secrete the IFN- γ that triggers isotype switching in TI-2-induced IgG production.

In the present study, we examined the *in-vitro* effect of human iNKT cells on Ig production by human B cells stimulated via cross-linking of their Ag receptors, which mimics BCR engagement by TI-2 Ags. We found that co-culture with iNKT cells reduced IgM production but increased IgG production by B cells stimulated via cross-linking of BCRs, and that this activity was higher in DN iNKT cells than in CD4 iNKT cells. These findings suggest that iNKT cells may contribute to the class-switching from IgM to IgG that occurs upon stimulation with TI-2 Ags.

2. Materials and Methods

2.1. Ethical Statement

All experimental protocols described in this study were reviewed and approved by the Ethics Committee for Human Experimentation at Tohoku University, Sendai, Japan (approval numbers: 2012-1-20, 2013-1-496).

2.2. Ab and Reagents

Alpha-galactosylceramide (α -GalCer; KRN7000) was purchased from Funakoshi (Tokyo, Japan). Recombinant

human (rh) IL-2 was from PeproTech (Rocky Hill, NJ, USA). Polyclonal hIgM and hIgG were from Rockland Immunochemicals (Limerick, PA, USA). Anti-human IgM, anti-human IgG, HRP-conjugated anti-human IgM and HRP-conjugated anti-human IgG for ELISA were from Acris Antibodies (San Diego, CA, USA). Goat F(ab')₂ anti-hIgM and rabbit F(ab')₂ anti-goat IgG for B cell stimulation were from Beckman Coulter (Fullerton, CA, USA). Anti-V α 24J α 18 (Clone; 6B11), anti-V β 11 (C21), isotype-matched control and 7-amino-actinomycin D (7-AAD) were from BD Pharmingen (San Diego, CA, USA). Anti-CD4 (RPA-T4) and anti-CD8 (RPA-T8) were from eBioscience (San Diego, CA, USA). Memory B cell isolation kit was from Miltenyi Biotec (Bergisch Gladbach, Germany).

2.3. Generation of V α 24J α 18⁺ Invariant NKT Cells

Human iNKT cells were separated from peripheral blood mononuclear cells (PBMCs) obtained from peripheral blood of healthy volunteers as described previously [18]. After 15 days of expansion, CD4⁺CD8⁻ (CD4) and CD4⁻CD8⁻ [double negative (DN)] iNKT subsets were sorted using a FACSAria cell sorter (Becton Dickinson, San Diego, CA, USA). The CD4 and DN iNKT cells (2×10^6 cells/well) were stimulated with irradiated allogenic PBMC (1×10^7 cells/well) prepulsed for 5 h with α -GalCer (100 ng/ml) in RPMI 1640 medium supplemented with 10% human serum, 100 U/ml penicillin G, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 25 mM HEPES containing 20 U/ml rhIL-2. From day 3 to day 9, cells were split into two fractions once or twice daily. The cultures were expanded by adding medium containing rhIL-2. On day 11 or 12, expanded cells were collected and used as iNKT cells. The surface phenotypes of expanded iNKT cells were identified by flow cytometry (FACSCant II; BD Biosciences). The presence of dead cells was excluded by running parallel 7-AAD-stained samples. After one or two passages of each primary cell culture, the remaining cells were used in the experiments.

2.4. Human Peripheral Blood B Cells

PBMCs were isolated from heparinized blood of one healthy adult volunteer by standard density gradient concentration over Ficoll-Paque PLUS (GE Healthcare Life Sciences, Piscataway, NJ, USA). Interface PBMCs were pelleted, washed twice, and resuspended in MACS buffer (Miltenyi Biotec). The naïve (CD19⁺CD27⁻) and memory phenotype (CD19⁺CD27⁺) B cells were isolated from PBMCs by a memory B cell isolation kit according to the manufacturer's protocol.

2.5. NKT and B Cell Cultures

The B cells used in the current assays were derived from a single donor. Initially, CD27⁻ and CD27⁺ B cells (2.5×10^4 cells/well) were stimulated with goat F(ab')₂ anti-human IgM (1 μ g/ml) for 15 min on ice. The B cells were washed three times with culture medium, and then co-cultured with rabbit F(ab')₂ anti-goat IgG (3 μ g/ml) in the presence or absence of CD4 or DN iNKT cells (2.5×10^3 - 2.5×10^4 cells/well) for five days. The culture supernatants were stored at -80°C until assayed for immunoglobulins by ELISA.

2.6. Measurement of Total IgG and IgM

The quantities of IgM and IgG in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Nunc A/S, Roskilde, Denmark) were coated with 250 ng/ml of anti-human IgM or 192 ng/ml of anti-human IgG Ab in PBS for 1 h at 37°C , and blocked with 1% FCS PBS at 4°C overnight. Prior to testing, samples were diluted with culture medium supplemented with 0.05% Tween 20. Next, serial two-fold dilution of hIgM or hIgG to 1:1024 was performed arbitrarily; resulting solutions were added to the wells and incubated at room temperature for 2 h. HRP-conjugated anti-human IgM or IgG antibodies diluted with 1:4000 were used as detection Ab. The concentrations of IgM and IgG were determined based on the absorbance at 450 nm. The detection limit was 0.2 ng/ml.

2.7. Statistical Analysis

Data are presented as mean values \pm standard deviation (SD). Differences between the two groups were tested using two-tail analysis in an unpaired Student's *t*-test. Differences among three or more groups were tested using ANOVA with post-hoc analysis (Student-Newman-Keuls test).

3. Results

3.1. Human CD4⁺CD8⁻ (CD4) and CD4⁻CD8⁻ (DN) V α 24 iNKT Cells

To investigate the functional differences between the human iNKT subsets in terms of immunoglobulin production against TI-2 Ags, we initially expanded α -GalCer-specific CD4 and DN iNKT cells separated from healthy individuals. More than 94% - 97% of the cultured iNKT cells, including both CD4 and DN subsets, expressed an invariant TCR, consisting of V α 24J α 18 CDR3 loop and V β 11. The expanded-CD4 iNKT cells were CD4⁺ CD8⁻ at 98% - 99% and V α 24J α 18⁺ CD4⁺ at 92%. By contrast, 98% - 99% of the DN iNKT cells were negative for expression of CD4 and CD8, whereas 97% of these cells expressed V α 24J α 18 (data not shown).

3.2. iNKT Cell-Induced Enhancement of Immunoglobulin Production by B Cells upon Stimulation with Antigen Receptor-Engagement

To investigate the effect of co-culture with iNKT cells on Ig production by B cells activated via cross-linking of BCRs, B cells were stimulated with anti-IgM Ab in the presence or absence of CD4 or DN iNKT cells, and the production of IgM and IgG in the culture supernatants was analyzed. As shown in **Figure 1(a)**, B cells produced large quantities of IgM under BCR cross-linking alone, whereas the addition of CD4 iNKT cells resulted in the reduction of IgM production in a dose-dependent manner. A similar pattern was observed upon co-culture with DN iNKT cells (**Figure 1(c)**). By contrast, IgG production by B cells was not clearly increased when activated via BCR cross-linking alone (**Figure 1(b)** and **Figure 1(d)**). The synthesis of IgG by B cells stimulated via cross-linking of BCRs was significantly enhanced by co-culture with CD4 and DN iNKT cells in a dose-dependent manner. In addition, this activity was much higher in DN iNKT cells than in CD4 iNKT cells (**Figure 1(b)** and **Figure 1(d)**).

3.3. Role of iNKT Cells in Immunoglobulin Production by Naïve and Memory B Cells upon Stimulation with Antigen Receptor-Engagement

IgM⁺CD27⁺ memory B cells in PBMCs play an important role in anti-pneumococcal polysaccharide IgG pro-

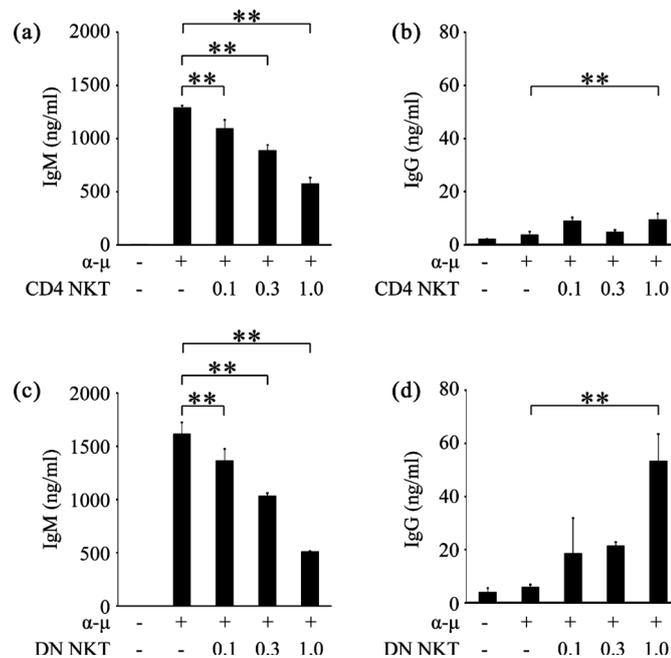


Figure 1. Effect of co-culture with iNKT cells on IgM and IgG production by B cells stimulated with anti-IgM Ab. B cells were stimulated with anti-IgM Ab in the presence or absence of CD4 iNKT cells or DN iNKT cells for five days, and the concentrations of Ig in the culture supernatants were measured. IgM (a) and IgG (b) production by B cells co-cultured with CD4 iNKT cells; IgM (c) and IgG (d) production by B cells co-cultured with DN iNKT cells. Similar results were obtained in two independent experiments. α - μ , anti-IgM Ab. **, $p < 0.01$.

duction in humans [19]. Therefore, to address the effect of co-culture with iNKT cells on Ig production by naïve (CD27⁻) and memory (CD27⁺) B cells, we separated B cells into two subsets according to the expression of CD27 and stimulated each subset with anti-IgM Ab in the presence or absence of CD4 or DN iNKT cells. As shown in **Figure 2(a)** and **Figure 2(c)**, both CD27⁺ and CD27⁻ B cells produced similar levels of IgM upon BCR cross-linking, and IgM production by both subsets was significantly reduced when co-cultured with either CD4 or DN iNKT cells. The cross-linking of BCRs induced low levels of IgG production by naïve and memory B cells in the absence of iNKT cells, and IgG production by CD27⁺ B cells was significantly higher than that by CD27⁻ B cells. In addition, IgG production by naïve and memory B cells was significantly enhanced when they were co-cultured with either CD4 iNKT cells or DN iNKT cells; this enhancement effect was much stronger with DN iNKT cells than with CD4 iNKT cells (**Figure 2(b)** and **Figure 2(d)**).

4. Discussion

In the present study, we evaluated the effect of co-culture with iNKT cells on Ig production by B cells upon stimulation via crosslinking of BCRs. Our data demonstrated that CD4 iNKT cells and DN iNKT cells accelerated the isotype switching from IgM to IgG, as shown by decreased IgM and increased IgG, in both naïve and memory B cells. DN iNKT cells accelerated this response even further than CD4 iNKT cells did. These results suggest that activation of iNKT cells may serve as a potent adjuvant, eliciting TI-2 Ag-induced IgG production in the development of more effective vaccination strategies for prevention of the infectious diseases caused by encapsulated bacteria.

Kobrynski and coworkers [16] demonstrated that TI-2 Ag-specific IgG production was completely abrogated in CD1d- or $\beta 2$ -microglobulin-deficient mice, suggesting that NKT cells may potentially promote IgG production in response to TI-2 Ags. In addition, we previously reported that activation of iNKT cells by α -GalCer increased IFN- γ -producing NKT cells, and that this increase was correlated with enhanced production of the poly-

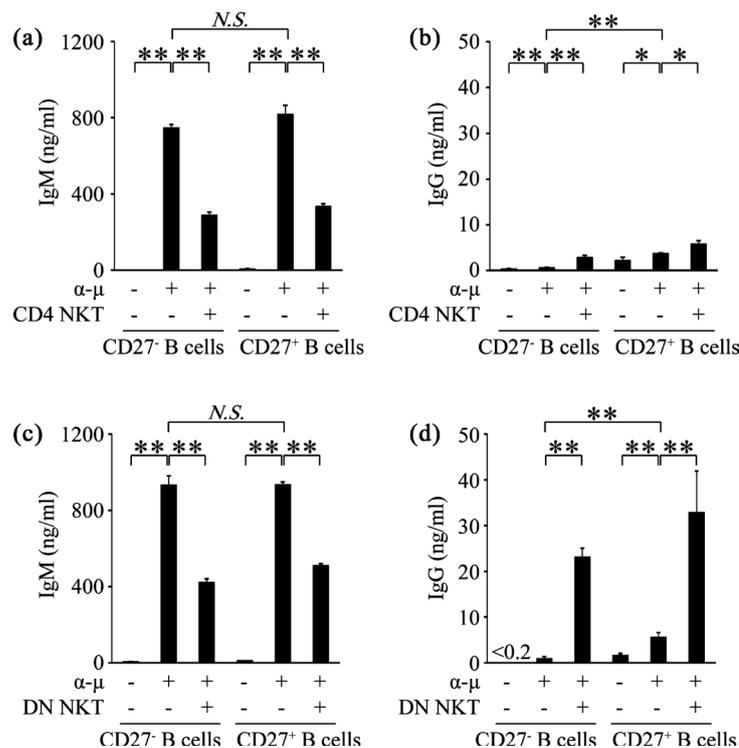


Figure 2. Effect of co-culture of iNKT cells on IgM and IgG production by naïve and memory B cells stimulated with anti-IgM Ab. CD27⁺ or CD27⁻ B cells were stimulated with anti-IgM Ab in the presence or absence of CD4 iNKT cells or DN iNKT cells for five days, and the concentrations of Ig in the culture supernatants were measured. IgM (a) and IgG (b) production by B cells co-cultured with CD4 iNKT cells; IgM (c) and IgG (d) production by B cells co-cultured with DN iNKT cells. Similar results were obtained in two independent experiments. α - μ , anti-IgM Ab. *, $p < 0.05$; **, $p < 0.01$; NS, not significant.

saccharide-specific IgG3 after immunization with pneumococcal polysaccharide vaccine in mice [4]. In our clinical study, immunization with anti-pneumococcal polysaccharide vaccine led to the production of serotype-specific IgG, which was correlated with an increase in DN iNKT cells in peripheral blood [5]. Thus iNKT cells are suggested to play an important role in the production of serotype-specific IgG after immunization with TI-2 Ags.

Co-culture with iNKT cells accelerated the production of IgG by B cells upon stimulation with BCR cross-linking without any exogenously added agonists of iNKT cells. In addition, α -GalCer did not induce any further increase in IgG production promoted by iNKT cells alone (data not shown). In earlier studies by Galli *et al.* [20], iNKT cell-induced promotion of B cell activation was demonstrated to depend on CD1d expressed on a variety of B cell subsets and to be delivered in the absence of α -GalCer [21]. These findings suggest that iNKT cells may recognize some endogenous ligand presented in context with CD1d on B cells, although the responsible molecule remains to be identified.

IgG production by B cells was more dramatically accelerated by DN iNKT cells than by CD4 iNKT cells. While CD4 iNKT cells have the potential to produce large amounts of Th2 cytokines such as IL-4 and IL-13, DN iNKT cells have a Th1-biased profile, enabling increased IFN- γ production and prominent expression of NK lineage receptors [15] [22]-[24]. In addition, chemokine receptors such as CCR6 and CXCR6 are preferentially expressed on DN iNKT cells rather than CD4 iNKT cells [22] [25], though the latter are the more common type among Th1 cells [26]. CD4 co-receptor potentiates the activation of human CD4 iNKT cells by engaging CD1d molecules [24]. Previously, Galli and co-workers [20] demonstrated that, compared to DN iNKT cells, human CD4 iNKT cells induced higher levels of IgM and IgG production in α -GalCer-pulsed B cells. In that study, B cells were considered to receive activation signals during cognate interaction with activated iNKT cells without BCR cross-linking. The current study differed from theirs in terms of the primary B cell activation method. Thus the phenotypic and functional properties of iNKT cells may be associated with IgG production by B cells stimulated with TI-2 Ags, though further investigation is required to define the molecular mechanism mediating the functional difference between CD4 iNKT cells and DN iNKT cells in T cell-independent Ig production.

TI-2 Ags are reported to generate memory B cells, although they do not elicit the Ab booster response or the germinal center formation following secondary immunization [27]. Moens and coworkers [19] reported that CD19⁺CD27⁺ IgM⁺ B cells were predominantly associated with an anti-polysaccharide IgG response after pneumococcal polysaccharide vaccination. In keeping with these previous observations, in the current study, cross-linking of BCRs induced the production of IgG by CD27⁺ (memory type) B cells at a higher level than that by CD27⁻ (naïve) B cells in the absence of iNKT cells. Yet iNKT cells promoted IgG production not only by memory B cells but also by naïve B cells. In a previous study by Bai and co-workers [28], iNKT cell activation through cognate interaction with dendritic cells induced isotype switching by B cells and promoted long-term memory response to pneumococcal capsular polysaccharides. In addition, CD4 iNKT cells and DN iNKT cells are reported to promote the proliferation of naïve and memory B cells derived from peripheral blood *in vitro* [20]. Thus, iNKT cells might be involved in the potentiation of IgG production by naïve and memory B cells upon stimulation with TI-2 Ags.

Maddur and coworkers demonstrated that B cells activated via BCR cross-linking enhanced the expression of OX-40L and co-stimulatory molecules such as CD80, CD86 and CD40 on DCs [29] [30]. In our previous study, DCs with increased expression of OX-40L caused NKT cells to produce substantial levels of IFN- γ [31]. Considered collectively, B cells activated by TI-2 Ags may amplify IFN- γ production by iNKT cells through the enhanced Th1 response induced by DCs *in vivo*.

5. Conclusion

In conclusion, we demonstrated that iNKT cells promoted the production of IgG by human CD27⁺ and CD27⁻ B cells upon stimulation via cross-linking of BCRs and that IgG production was more strongly promoted by DN iNKT cells than by CD4 iNKT cells. The present study provides important implications for understanding the contribution of iNKT cells to IgG production by TI-2 Ag-stimulated B cells, which is expected to be helpful in the development of more effective vaccination strategies for prevention of pneumococcal infection.

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Conflicts of Interest

The authors have no financial conflicts of interest.

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