

Original article

Preferential expression of IgG2b in nose draining cervical lymph nodes and its putative role in mucosal tolerance induction

Induction of intranasal tolerance prevents the body from eliciting unwanted immune responses against harmless antigens that enter the body through the nasal mucosa. To study the intrinsic capacities of the cervical, nose draining lymph nodes (CLN), which are essential for tolerance induction, genes that are differentially expressed in CLN and not in peripheral lymph nodes (PLN) were characterized. The gene that is predominantly overexpressed in CLN codes for IgG2b. This is confirmed by a higher percentage of IgG2b⁺ B220⁺ cells in CLN compared with any PLN. However, this predominance of IgG2b-positive B cells in the CLN is not specific for the lymph node itself but rather determined by the region drained by lymph nodes at the cervical site, as transplanted PLN at these locations show a comparable predominance. It was demonstrated that IgG2b, when compared with IgG1, led to differential activation of dendritic cells (DC) through Fc receptor signalling. The results point to a unique local combination of cells and factors in the nose draining CLN leading to highly specialized immune reactivity. The results point out that predominance of a distinct IgG isotype in a lymphoid environment may lead to highly specialized immune reactivity.

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Through the induction of mucosal tolerance the body is prevented from harmful inflammatory reactions against nondangerous proteins such as food components. This naturally occurring immunological phenomenon has been described for the mucosae of the upper and lower airways and the gastrointestinal tract (1–3) and involves clonal deletion and anergy of T cells, but also active T-cell mediated suppression (4, 5). The latter is especially found when tolerance is induced via the nasal mucosa, and leads to the formation of regulatory T cells (6, 7), capable of expanding their suppressive capacity by infectious tolerance (8). The nasal site may therefore be favourable when therapeutic applications of mucosal tolerance are considered to prevent allergies and autoimmune disorders, as both IgE-production and delayed type hypersensitivity (DTH) can be efficiently down-regulated by nasal tolerance (4, 9, 10).

The initial steps leading to the induction of tolerance after antigen is deposited on the mucosal surfaces are still

largely unknown. In recent studies, we have shown that nasal deposition of antigen leads to rapid expansion of T cells with a regulatory, suppressive function (6), but the precise mechanism of suppression is still unclear.

In case of nasal tolerance, we have demonstrated in mice the absolute requirement for the organized lymphoid tissue of the nose draining lymph nodes (10). Not only was the presence of cervical lymph nodes (CLN) necessary for tolerance induction, but this function could not be taken over by any other lymph node when transplanted to the cervical site (10).

Consequently, neither the uptake of antigen through the mucosal surface nor the uptake and transport of antigen by the antigen presenting cells from the mucosal surface to the draining CLN seems to determine whether tolerance is induced or not. Rather, intrinsic capacities of CLN, the superficial cervical and internal jugular in particular, are instrumental for the mode of antigen presentation by specialized antigen presenting cells to T cells, eventually leading to active immunological tolerance.

To further understand the mechanisms of these regulatory processes we studied the differences between the

Abbreviations: CLN, cervical lymph nodes; PLN, peripheral lymph nodes.

CLN and the peripheral lymph nodes (PLN). To this, the gene expression profile of the two types of lymph nodes was compared. cDNA of PLN was subtracted from cDNA of CLN and the differences in gene expression were analysed. A major difference in expression was observed for IgG2b. This preferential isotype expression was confirmed by higher number of IgG2b producing B cells in CLN and emphasizes the specific role of the local microenvironment of the CLN in determining the directions of initiated immune responses, at the level of tolerance induction and specific isotype switching.

Materials and methods

Mice

BALB/c mice were obtained from Harlan (the Netherlands) and kept in our animal facility under routine laboratory conditions. All experiments performed were approved by the animal experiments committee of the VUMC.

Subtractive hybridization

Subtraction of PLN cDNA from CLN cDNA was performed according to the manufacturer's protocol for polymerase chain reaction (PCR)-select (Clontech, Palo Alto, CA). In brief, total RNA was isolated from superficial CLN and PLN (axillary, brachial, popliteal, inguinal) using the RNAeasy kit (Qiagen, Hilden, Germany). mRNA was purified from total RNA using oligo-dT beads (Dyna, Oslo, Norway). Double-stranded cDNA was synthesized from the purified mRNA and digested with *RsaI*. Adaptors were ligated to cDNA of the CLN and hybridization performed in the presence of an excess of PLN cDNA according to the manufacturer's protocol (Clontech). The resulting difference product was selectively amplified due to the principle of suppression PCR and cloned into the pGEM-T-easy vector (Promega, Madison, WI).

RT-PCR for γ -immunoglobulins, TGF- β 1 and IL-6

Total RNA was purified from lymph nodes or sorted B cells using Trizol (Gibco BRL, Breda, the Netherlands). cDNA was synthesized from total RNA using oligo-dT primer (Gibco BRL) and mouse mammary leukemia virus reverse transcriptase (RT; Gibco BRL). To perform semiquantitative PCR for the different γ -immunoglobulins the constant regions of γ -immunoglobulins were aligned using the ClustalW algorithm (Hinxton, UK). γ -Immunoglobulin specific primers were designed across different constant region exons resulting in these primers:

IgG1: 5'-CCAGGGATTGTGGTTGTA-3',
IgG1: 3'-GCAGGGAAAGCTGCACT-5'
IgG2b: 5'-TCCATGCAAGGAGTGTC-3',
IgG2b: 3'-GCATGAGAAGGAATCTGT-5'
IgG3: 5'-CTAGAATACCAAGCCCA-3',
IgG3: 3'-GGACATTTGTTACGAG-5'.

For TGF- β 1 and IL-6 the following primers were used:

TGF- β 1: 5'-CGGGTGGCAGGCGAGAG-3',
TGF- β 1: 3'-TCAGCAGCCGGTTACCAAGG-5'
IL-6: 5'-GTTCTCTGGGAAATCGTGGA-3',
IL-6: 3'-TGTAACAAGGTAGCTATGG-5'.

Actin was used to quantify the amount of cDNA:
actin: 5'-TGGATTCTGTGGCATCCATGAAAC-3',
actin: 3'-TAAAACGCAGCTCAGTAACAGTCCG-5'.

For semiquantitative PCR the templates were diluted from cDNA in fivefold dilution steps. Cycling conditions on the thermocycler (MJ-research, Watertown, MT) were 94°C for 30 s, 50 (IgGs) or 62°C (TGF- β 1) for 30 s, 72°C for 45 s for 40 cycles and for IL-6, 94°C for 30 s, 56°C for 2 min, 72°C for 3 min, for 10 cycles followed by 94°C for 30 s, 56°C for 1 min, 72°C for 3 min, for 30 cycles.

Cell staining and FACS analysis for immunoglobulins/cell sorting of lymphocytes

Single cell suspensions of lymph nodes were made by forcing the cells through a nylon mesh. Cells were washed in RPMI containing 2% new born calf serum. Antibodies used were biotin-labelled rabbit anti-mouse IgM, IgA, IgG2a, IgG2b (Zymed, San Francisco, CA) in combination with streptavidin-PE (Vector, Burlingame, CA). To gate for B cell samples were additionally stained with 6B2 (α -B220), coupled to Alexa488 (Molecular Probes, Eugene, OR). 7AAD (Molecular Probes) was used to stain for dead vs live cells.

For RT-PCR lymphocytes were isolated from lymph nodes as described above. Purified B cells (>90%) and T cells (>95%) were obtained in a single step by sorting lymphocytes stained with 6B2-Alexa488 (α -B220) in the fluorescence 1 channel and GK1.5-PE (α -CD4; Pharmingen, San Diego, CA) and 53-6.7-PE (α -CD8, Pharmingen) in the fluorescence 2 channel on a FACStar Plus (Becton Dickinson, Mountain View, CA).

Dendritic cells

D1 dendritic cells (DC), a kind gift of Dr P. Ricciardi-Castagnoli, were cultured as described previously (11). In short, D1 cells were seeded at $1.5\text{--}2.0 \times 10^6$ cells per dish in Iscove's modified Dulbecco medium (IMDM) supplemented with R1 medium to obtain a final concentration of 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF). The cells were harvested after 3 or 4 days of culture and used for propagation or subsequent experiments.

Dendritic cell staining, FACS analysis, and antibodies

Cultured DC or single cell suspensions of lymph nodes prepared by using a nylon mesh were washed in phosphate-buffered saline (PBS) containing 2% FCSi and 0.01% Sodium azide (FACS-buffer; Merck, Amsterdam, the Netherlands). Antibodies used were anti-CD86 (clone GL-1), anti-CD40 (clone 3/23), FITC-conjugated anti-MHCII (clone M5/114), PE-conjugated anti-CD80 (clone 1G10), biotin-conjugated anti-CD11c (clone N418), and PE-conjugated anti-IL-12 (p40). Subsequently, the cells were washed three times with FACS-buffer and when conjugate staining was necessary the cells were incubated on ice for 30 min with PE-conjugated donkey anti-rat immunoglobulin (Jackson Labs, Ann Arbor, MI) or streptavidin-PE (Vector). To gate for B cells in lymph node suspensions samples were additionally stained with 6B2 (α -B220), coupled to Alexa488 (Molecular Probes). 7AAD (Molecular Probes) was used to stain for dead vs live cells.

For staining of intracellular IL-12 the Cytofix/Cytoperm Plus kit with GolgiStop™ (BD Biosciences, Alphen aan de Rijn, the Netherlands) was used according to the manufacturer's instructions.

After incubation the cells were washed, resuspended in FACS-buffer and fluorescence was measured using a FACScan™

(Beckton Dickinson). Per sample 10 000 events were analysed. Cells that had been incubated with conjugate alone served as negative control.

The K9361 hybridoma specific for the Ly17.1 allele of the Fc γ RIIB (12) was obtained from Dr Marc Daeron and used as affinity purified immunoglobulin.

In vitro stimulation with IgG2b, IgG1 and blocking of Fc γ RIIB

Twenty-four well plates (Greiner, Alphen aan de Rijn, the Netherlands) were coated with either purified mouse IgG2b anti-TNP or purified mouse IgG1 anti-TNP (Pharmingen) at 10 μ g/ml in PBS at 37°C. After a 60 min incubation the coating solution was removed. D1 DC were washed and seeded in the wells at 1×10^6 /ml in the presence or absence of lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Difco, Detroit, MI) at a concentration of 1 or 10 ng/ml. For blocking of Fc γ RIIB during stimulation D1 cells were harvested, washed and preincubated in suspension with K9361 monoclonal antibody (mAb) or isotype control mAb ED6, with no known reactivity against mouse tissues, at a concentration of 10 μ g/ml in PBS at room temperature during 60 min. After incubation the cells were centrifuged and resuspended in IMDM, seeded in the immunoglobulin-coated wells at 1×10^6 /ml in the presence or absence of 10 μ g/ml K9361 or ED6. After a 60 min of incubation the cells were stimulated with LPS. The cells were cultured at 37°C and 5% CO₂ and after 24 h the supernatant was collected, stored at -80°C, and the cells were recovered for further analysis.

IL-12 p40 determination

The concentration of IL-12 p40 in the culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA; Biosource, Nivelles, Belgium), which was performed according to the instructions of the manufacturer.

Statistical analysis

Significance was analysed using an unpaired *t*-test. A *P*-value of <0.05 was considered significant.

Results

Selection for genes that are preferentially expressed in the cervical lymph node

In order to identify genes that are unique to the CLN and thus potentially involved in induction of tolerance, subtractive hybridization of cDNA from CLN *vs* cDNA of PLN was performed to enrich for those genes that are preferentially expressed in the CLN. Poly A⁺-RNA was isolated from CLN and pooled PLN and after subtractive hybridization the resulting difference product of the two types of lymph nodes was cloned into a T/A cloning vector. A total of 450 randomly selected clones, putatively preferentially expressed in the CLN, were sequenced. In the resulting subtractive library of 450 clones the IgG2b gene was represented by 24 clones, whereas the next most abundant genes were represented by six clones for each gene. The abundance of the IgG2b

clones was a strong indication for differential gene expression of IgG2b.

Increased expression of IgG2b in the cervical lymph node

The differential expression of IgG2b in CLN *vs* PLN, was confirmed at mRNA level. As the genes encoding the different γ -immunoglobulins are highly homologous, semiquantitative PCR was preferred over Northern blotting for the detection of IgG2b expression levels.

Semiquantitative PCR on total lymph node material showed that IgG2b was expressed at 125-fold higher levels in the CLN relative to the PLN (Fig. 1A). Compared with expression of IgG2b, expression of IgG1 and IgG3 mRNA was moderately increased in the CLN (Fig. 1B).

The increase in IgG2b expression in the CLN could be due to (i) higher percentages of total B cells, (ii) a specific increase in the number of IgG2b-positive B cells, or (iii) increased synthesis of IgG2b per cell.

To discriminate between these possibilities the percentages of B cells in CLN and PLN were determined and the contribution of the various isotypes was analysed. As shown in Fig. 2, the percentage of total B cells in CLN and PLN was not different, as determined by B220 expression. However, in CLN there was a significantly higher proportion of B cells expressing the IgG2b isotype (Fig. 2), which was not found for IgG2a and IgA, making it unlikely that this was due to more general activation of CLN compared with peripheral nodes. This was confirmed by isolation of B220⁺ B cells from the various lymph nodes using FACS sorting (Fig. 3). Using cDNA

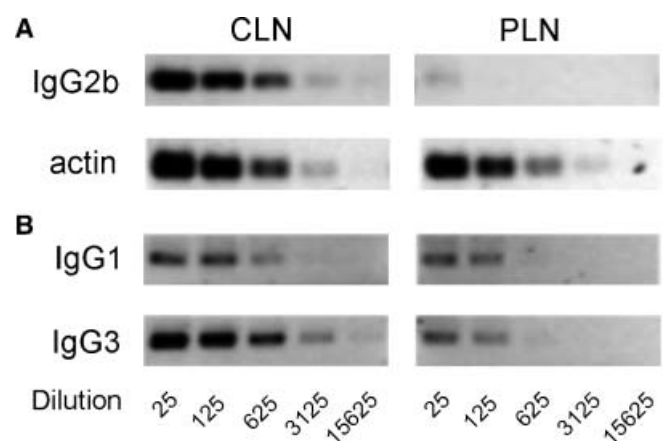


Figure 1. Increased expression of IgG2b mRNA in cervical lymph nodes. Cervical and peripheral lymph nodes were isolated and expression of mRNA was analysed by semiquantitative polymerase chain reaction (PCR). Actine was used for normalization of mRNA levels. (A) expression of IgG2b, (B) expression of IgG1 and IgG3.

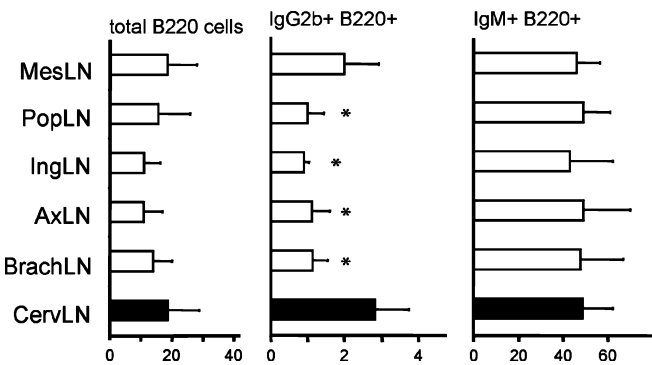


Figure 2. Cervical lymph nodes contain relatively more IgG2b⁺ B220⁺ cells than peripheral lymph nodes (LN). Lymphocytes were isolated from LN of untreated mice and stained for B cells (B220) and immunoglobulin isotype. 7AAD was used to stain dead cells. The isotype-specific cells were gated for B220 and living cells. (A) percentage of B220⁺ cells, (B–E) percentage of isotype-specific B cells, (B) IgG2b, (C) IgG2a, (D) IgM and (E) IgA. Lymph node annotations are: MesLN, mesenteric LN; PopLN, popliteal LN; IngLN, inguinal LN; AxLN, axillary LN; BrachLN, brachial LN; CervLN, cervical LN. The results are from a representative experiment of four performed. *Statistical significance: $P < 0.05$.

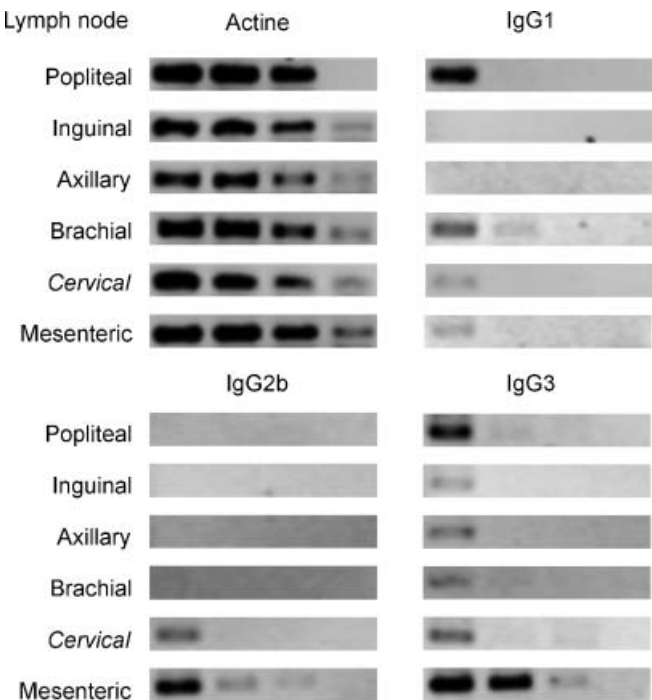


Figure 3. B220⁺ cells from mucosa draining lymph nodes produce more IgG2b mRNA than B220⁺ cells from peripheral lymph nodes. B220⁺ cells were sorted by flow cytometry from cervical and peripheral lymph nodes, and analysed with semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for the expression of IgG2b, IgG1, IgG3. Actin was used for normalization of mRNA levels.

from these cells in a semiquantitative PCR IgG2b transcripts could not be detected in B cells from PLN, but B cells from the CLN as well as the mesenteric lymph node expressed IgG2b. Transcripts for IgG1 and IgG3 were also found, but not preferentially expressed in the mucosa draining lymph nodes (Fig. 3). The abundant IgG2b expression in CLN compared with PLN is therefore directly correlated to a higher percentage of IgG2b-positive B cells.

Upstream effects that lead towards IgG2b synthesis

Several switch factors-like TGF- β 1 and IL-6, are associated with isotype switching and differentiation of B cells to the synthesis of IgG2b antibodies. Especially TGF- β 1 is considered as a main switch factor for IgG2b. TGF- β 1 can be produced by many cell types, including T cells. We therefore first analysed whole lymph node preparations for the production of TGF- β 1 by semiquantitative PCR. However, no differences were found in whole tissue cDNA from CLN vs PLN (results not shown). When T lymphocytes from the two types of lymph nodes were sorted and analysed for their level of TGF- β 1 by semiquantitative PCR no differences in expression could be found between T cells from CLN and PLN (results not shown).

Transcripts for IL-6, a cytokine also associated with differentiation of B cells and the isotype switching into IgG2b were readily found in all lymph nodes but no differences were found between the different types of lymphoid tissue (results not shown).

Effects of tolerance induction on dendritic cells

Crucial for the induction of tolerance in the nose draining CLN are the antigen presenting DC. Careful analysis of the expression of co-stimulatory molecules and levels of MHC and CD11c did not reveal any major differences in these cell types between CLN and PLN (results not shown). However, within 24 h after nasal instillation of antigen (OVA) a significantly larger population DC from CLN produced IL-12, compared with DC from peripheral nodes (Fig. 4), suggestive for local factors influencing the mode of antigen presentation.

To see if the increased expression of IgG2b in CLN could underly this phenomenon the effects of IgG2b on the activation of DC through Fc receptor activation were studied. Hereto D1 cells were cultured in plates coated with IgG2b or IgG1 in the presence of low-dose LPS. After 24 h the cells were analysed for expression of co-stimulatory molecules by FACS and the production of IL-12 was determined.

Both immobilized IgG2b- and IgG1-stimulated DC activation as evidenced by increased expression of the co-stimulatory molecules CD80, CD86 and CD40 (Fig. 5). While IgG2b and IgG1 enhanced the expression of co-stimulatory. In the presence of low-dose LPS it was

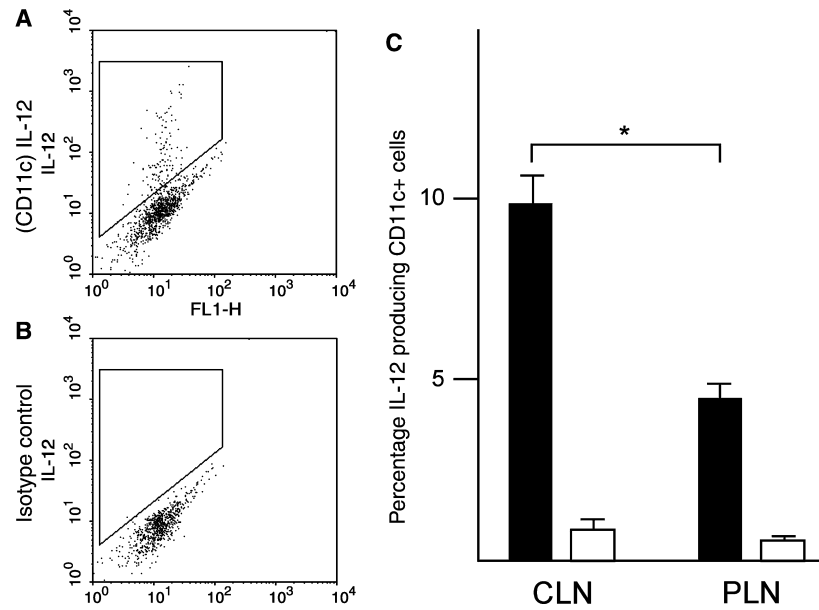


Figure 4. Dendritic cells produce IL-12 p40 after nasal antigen administration. BALB/c mice received a single intranasal dose of 400 µg ovalbumin in 10 µl saline or saline only and 24 h later cervical and peripheral (brachial and inguinal) lymph nodes were suspended and stained for CD11c and intracellular anti-IL-12 (p70). The number of CD11c+; IL-12 producing dendritic cells in the lymph nodes was enumerated by flow cytometry. (A and B): flow plots of IL-12 staining of CD11c+ gated cells. (C): histograms of the percentage of dendritic cells producing IL-12 in the various lymph nodes. CLN, cervical lymph nodes; PLN, peripheral lymph node. *Statistical significance: $P < 0.05$.

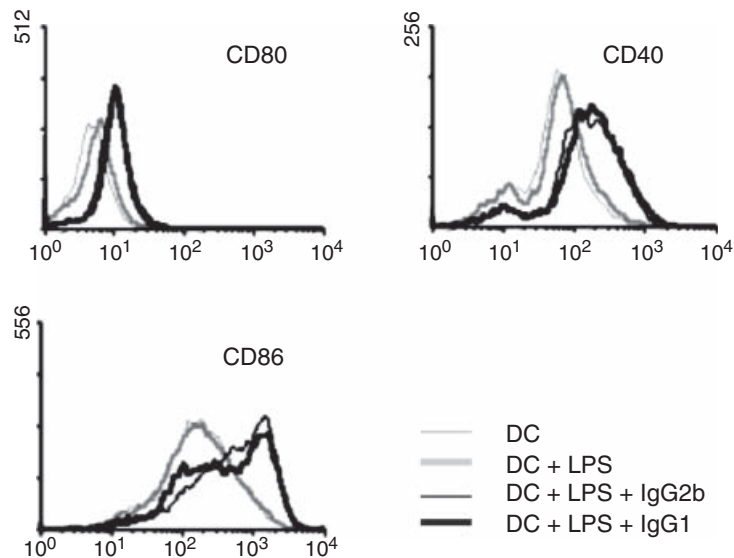


Figure 5. Fc γ RIIB regulates a balance in accessory molecule expression by dendritic cells (DC). Dendritic cells were cultured in 24-well plates coated with purified, endotoxin low, mouse IgG1 anti-TNP 10 µg/ml, mouse IgG2b anti-TNP 10 µg/ml in phosphate-buffered saline (PBS) or PBS as a control at 37°C. After a 60 min incubation the cells were stimulated with lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 at a concentration of 10 or 100 ng/ml. The cells were cultured at 37°C and 5% CO₂ and after 24 h the cells were centrifuged, resuspended in IMDM and stained for co-stimulatory molecule expression and analysed by flow cytometric analysis. Histograms are representative of $n = 3$ experiments.

found that IgG2b seemed clearly less potent in inducing IL-12 p40 secretion than IgG1 (Fig. 6). Immunoglobulin G-stimulated DC activation was enhanced with

increasing LPS concentrations, which may be due to up-regulation of the IgG receptors Fc γ R. As Fc γ RIIB is primarily expressed on resting DC and has been proposed

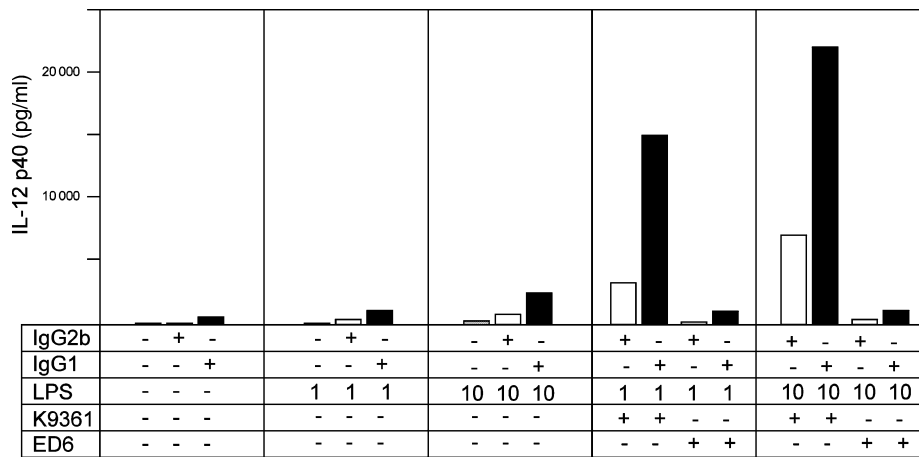


Figure 6. FcγRIIB regulates a balance in IL-12 secretion by dendritic cells (DC). Twenty-four-well plates were coated with purified, endotoxin low, mouse IgG1 anti-TNP 10 µg/ml, mouse IgG2b anti-TNP 10 µg/ml in phosphate-buffered saline (PBS) or PBS as a control at 37°C. After a 60 min incubation, the coating solution was removed and DC were seeded in the wells at 1 × 10⁶/ml. FcγRIIB signalling was neutralized by first preincubating the DC in suspension with K9361 monoclonal antibody (mAb) or isotype control ED6 mAb at a concentration of 10 µg/ml in PBS at room temperature during 60 min. After incubation the cells were centrifuged, resuspended in IMDM and seeded in the coated 24-well plates at 1 × 10⁶/ml in the presence or absence of 10 µg/ml K9361 or ED6. After a 60 min incubation the cells were stimulated with lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 at a concentration of 1, 10 or 100 ng/ml. The cells were cultured at 37°C and 5% CO₂ and after 24 h the supernatants of the cultures were collected and IL-12 concentration was determined using a sandwich enzyme-linked immunosorbent assay (ELISA). Values are representative for three experiments.

to mediate tolerizing effects, because of the inhibitory ITIM motif in its cytoplasmic tail, it was therefore assessed whether neutralization of this receptor altered IgG-mediated DC activation. Indeed, neutralization of FcγRIIB with the mAb K9361 during IgG stimulation dramatically enhanced IL-12 secretion by DC (Fig. 6).

Discussion

The nature of an immunological reaction is highly dependent on the organization and local conditions of the lymphoid microenvironment where the response is being initiated. Differential activation of antigen presenting cells underlies the skewing of Th1 and Th2 responses, and comparable regulation of cell activation may form the basis of tolerance induction. For tolerance induction through the nasal mucosa the basic, reticular framework of the cervical, nose draining lymph nodes is essential, as can be inferred from our previous transplantation experiments (10). The transplantation of lymph nodes is characterized by a complete loss of the mobile populations from the donor-tissue, such as DC and lymphocytes during the initial taking of the graft [(13) and manuscript in preparation]. Yet, only transplanted CLN and no other lymph node types can confer tolerance upon nasal antigen deposition. This indicates that the immigrating cell populations of haemopoietic origin are dictated by cells or factors in the microenvironment of the CLN to direct the antigen activation in a tolerogenic fashion,

possibly in combination with factors from the draining area.

It may well be that the specific conditions of the CLN required for tolerance induction favour the production of IgG2b. These conditions could be regulated in part by the mucosal drainage. This correlates with the fact that also in mesenteric lymph nodes and Peyer’s patches the percentage of IgG2b bearing cells is increased (Fig. 2), which could be related to the high amounts of TGF-β1 found associated with mucosal tissues, including airway epithelium (14). Several reports show a role for TGF-β1 in tolerance induction, either through activation of regulatory cells, or direct immunosuppression by this cytokine (15, 16). As a secondary consequence of these local TGF-β1 levels enhanced IgG2b switching may occur.

In spite of the active role of TGF-β1 in relation to tolerance and regulatory T cells we could not demonstrate any differences in TGF-β1 expression in CD4+ T cells from CLN. This could be related to the fact that T regulatory cells may comprise only a fraction of the lymph node cells, but could also come from the fact that lymph nodes are the induction site of these cells and that down-modulating cytokines such as TGF-β1 and IL-10 are used by matured effector cells residing in systemic tissues such as lamina propria and epithelium. In addition to certain local cytokines, IgG2b switching may also be favoured through the presence of certain types of bacterial antigens at mucosal sites. However, a specific association of IgG2b induction with certain antigenic determinants, as found for IgG3 and polysaccharide

(17) has never been demonstrated. There are indications that IgG2b production can be specifically enhanced by LPS, more than other isotypes, but this is debated and primarily based on *in vitro* data (17, 18).

Is there a relationship with high levels of IgG2b and tolerance induction in the mucosal lymph nodes? At first thought it is not logical that an effector molecule that will be produced relatively late in an immune response can have a role in determining the direction of an immune response. However, recent reports on intravenous injection of γ -globulin (IVIG) during allergy and autoimmune disease (19, 20), suggest that nonspecific immunoglobulin can down-regulate an immune response, e.g. by enhancing Fc γ RIIB expression and interfering in the balance between signalling via the inhibitory Fc γ RIIB and the stimulatory Fc γ RIII (21). Our experiments in which IgG2b and IgG1 can activate DC through Fc receptor-mediated signalling, show that immunoglobulins can exert regulatory functions independent of their relevant antigen. IgG2b, was less potent than IgG1 in inducing IL-12 p40 secretion suggesting the isotypes have differential immunoregulatory effects. IgG2b stimulation is largely mediated by signalling through the low affinity receptor Fc γ RIIB as seen after blocking with the K9361 antibody. The ITIM motif in the cytoplasmic tail of Fc γ RIIB can negatively modulate activation of cells upon binding of immune complexes. The Fc γ RIIB is the most widely distributed and abundant of the Fc γ receptors, expressed on macrophages, granulocytes, B cells and stimulated T cells (22). As complexes IgG2b, IgG2a and IgG1 can bind to Fc γ RIIB and recently it was reported that immune complexes can lead to maturation of human DC from monocytes, acting via cross-linking of Fc γ RIIB (23). This maturation was accompanied by a moderate increase of IL-10 production, but no IL-12 was produced, in line with other observations on the effects of immune complexes in human monocytes (24). Although in these reports the effects of different isotypes were not studied, it is clear that FcR-mediated signalling can influence the maturation state of DC, leading to more immunogenic or tolerogenic DC, the latter characterized by lack of IL-12 secretion and increased IL-10 production (25). Our findings that IgG2b is less well capable of inducing IL-12 production in DC (Fig. 5), together with the prominent production of the IgG2b isotype in mucosal

lymph nodes may therefore favour the maturation of tolerogenic DC. The specificity of the immunoglobulin in the complexes can therefore be irrelevant with respect to the antigen that is presented in a tolerogenic way.

Fc γ RIIB signalling has been shown to induce other regulatory factors such as prostaglandins (26), which also may lead to a tolerogenic antigen presenting cell (27). In addition to an effect on DC, IgG2b may directly affect stimulated T cells, which can up-regulate Fc γ RIIB upon activation (28). This may play a role in the induction of regulatory T cells.

Studies using mice mutated in their Fc γ RIIB have shown that this lack of the inhibitory arm of immune complex activation leads to elevated serum IgG levels and renders mice susceptible to induced autoimmune diseases (29, 30). Furthermore, it was demonstrated that the mechanism by which Fc γ RIIB deficiency results in autoimmunity was dependent on the loss of peripheral tolerance of the B cells involved (31).

Fc γ RIIB might also be present on nonhaematopoietic, stromal cells of the reticular framework, as described for stromal cells in the kidney (32). There, after binding of immune complexes to Fc γ RIIB, stromal cells will synthesize chemokines-like MCP-1 to recruit macrophages. When similar processes would occur in lymph nodes, it can be envisaged that, in combination with the specific expression of the mucosal addressin MAdCAM on high endothelial venules of CLN (10), locally different chemokine profiles assist in the recruitment and retention of specific lymphocyte subsets.

Together, it is clear that the nose draining lymph nodes form a unique microenvironment, both through the composition of the stromal elements that form the structural basis of these organs and are pertinent for tolerance induction, and through their draining site, which is important for the preferential switching to IgG2b. The stromal elements can exert their effects directly on both DC and T cells or only on one of the two types of cells. From the experiments shown here it is clear that the effects on B cell isotype switching are indirect.

Subtle differences at the level of the local microenvironment seem crucial for directions that immune responses take and further studies on the regulation of these *in vivo* differences will be important for applications of tolerance induction in disease.

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