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Effect of Toll-like receptor agonists on allergen-induced human basophil activation

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ανερμήνευτος, άπειρος, απερινόητος
απλανής οδηγός, αφανής
αυτός
ο άφωνος κόσμος

Αθανάσιος Αλεξανδρίδης

unexplicable, unlimitable, unthinkable
undisclosed but showing no false way
this
unvoiced world

*Athanassios Alexandridis**

* “Shadowplay (Σκιαμαχία)”, taken from the collection of poems “Terms of certainty (II)”, Athens 2004

Contents

Abbreviations used

C	Complement
CARD	Caspase-recruitment domain
CCR	Chemokine receptors
CD	Cluster of differentiation
CRTH2	Chemoattractant receptor homologous molecule expressed on TH2 cells
D	Donor
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
ELISA	Enzyme-linked immuno sorbent assay
GM-CSF	Granulocyte/macrophage-colony-stimulating factor
HRF	Histamine-releasing factor
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule
iE-DAP	γ-D-glutamyl-meso diaminopimelic acid
IFN	Interferon
IL	Interleukin
LBP	Lipopolysyccharide-binding protein
LFA-1	Leukocyte function-associated antigen-1
LPS	Lipopolysaccharide
LT	Leukotriene
LTA	Lipoteichoic acid

mAb	Monoclonal antibody
MDP	Muramyl dipeptide MurNAc-L-Ala-DisoGln
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor κB
NGF	Nerve growth factor
NOD	Nucleotide-binding oligomerization domain
PECAM-1	Platelet/endothelial cell adhesion molecule-1
PGN	Peptidoglycan
Poly(I:C)	Polyinosine-polycytidylic acid
PSGL-1	P-selectin glycoprotein ligand-1
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
SCF	Stem-cell factor
sLT	Sulfidoleukotriene
ssRNA	Single-stranded RNA
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TRAM	TRIF-related adaptor molecules
TRIF	TIR domain-containing adaptor inducing IFN-β

1. Introduction

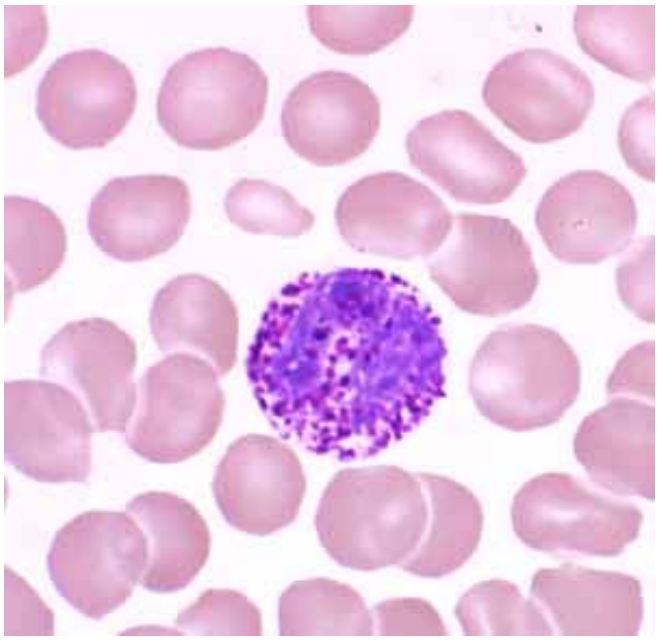
IgE, mast cells, basophils, and eosinophils constitute essential elements in allergic inflammation. Allergen-specific IgE, synthesized in response to allergens in the environment and in susceptible individuals, becomes fixed to high-affinity receptors on cellular membranes, especially of mast cells and basophils. If these receptor-bound IgE molecules are aggregated on reexposure to specific allergen, these mast cells and basophils produce mediators that result in the allergic response. Principal among the cells drawn to sites of mediator release is the eosinophil (88).

1.1. Basophils

The basophil was first described in the peripheral blood by Ehrlich more than one hundred years ago (Figure 1) (35). Basophils are granulocytes that are believed to represent a separate lineage from mast cells, despite the fact that the two cell types share many common features, such as high-affinity IgE receptor (FcεRI) expression, metachromatic staining, TH_2 cytokine expression, and histamine release. They are the rarest of leukocytes, normally accounting for less than one percent (1%) of those leukocytes found in blood (57). One marker of systemic effects of allergic disease is a stable basophilia in allergic subjects, and the number of circulating basophils increases even more (approximately 2-fold) during seasonal allergen expsoure (51, 92).

Figure 1. Basophil. A: Hematosin & Eosin stain (118), B: Human basophil isolated from the peripheral blood has electron-dense secretory granules, and a polylobed nucleus (x17,000) (33).

A



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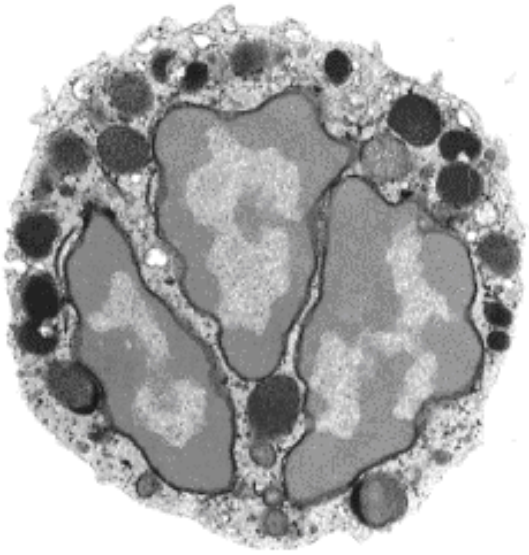


1.1.1. Morphology and phenotype

Basophils exhibit a segmented nucleus with highly condensed chromatin and are commonly identified by their metachromatic staining with basic dyes, such as toluidine blue. Two basophil granule-specific monoclonal antibodies, BB-1 and 2D7, have recently been developed, permitting the unambiguous identification of basophils in tissues and greatly furthering our understanding of the role of basophils in allergic diseases (25).

With the use of cell surface IgE or FcεRI, multicolor flow cytometry easily permits cell-surface marker analysis (15). With these and other techniques, basophils have undergone extensive phenotypic analysis to characterize their surface structures (19, 20, 109).

Basophils express a variety of cytokine receptors (CCR2, CCR3), complement receptors (CD11b, CD11c, CD35, CD88), prostaglandin receptors (CRTH2), and immunglobulin Fc receptors (FcεRI, FcγRII) (2, 16, 94). Human basophils were recently found to express high levels of TLR2 and TLR4 compared with other granulocytes (10, 94) raising the possibility of TLR involvement in mediating basophil responses.

The expression and function of a wide variety of adhesion molecules, including members of the integrin (α4β1, α5β1, LFA-1, Mac-1, p150,95, ad, α4β7), selectin (L-selectin, PSGL-1, sialyl Le, sialyl-dimeric Le), and immunoglobulin gene superfamilies (ICAM-1, ICAM-2, ICAM-3, PECAM-1), have also been demonstrated. Basophils express several peptidases, although their functions are unknown (16, 21). Flow cytometry is a reliable tool for monitoring basophil activation upon allergen challenge by detecting surface expression of degranulation/activation markers such as CD63 (a member of the tetraspanner family) (61) or CD203c (belonging to the

type II transmembrane protein family) (23). The expression of CD63 appears to correlate with degranulation (61, 79). Activation also results in increased expression of β2 integrins (CD11b, CD11c and αd), whereas L-selectin (CD62L), platelet-endothelial cell adhesion molecule-1 (CD31), and P-selectin glycoprotein ligand (CD162) are shed (17, 18, 106).

1.1.2. Development

Basophils develop from CD34+ pluripotent stem cells, differentiate and mature in the bone marrow, and then circulate in the periphery. Interleukin (IL)-3 is the dominant cytokine driving basophil differentiation and is sufficient to differentiate stem cells into basophils (59). The general consensus is that basophils represent a separate cell lineage from mast cells and differentiate from a common basophil-eosinophil precursor; this belief is supported by the derivation of mixed colonies of basophils and eosinophils from individual precursor cells (31).

1.1.3. Activation

As with mast cell, basophils expresse a complete and functional FcεRI receptor ($\alpha\beta\gamma_2$) (Figure 2), a cross-linking of which leads to basophil activation, granule exocytosis, and mediator release (60). C3a and C5a can also activate basophils through the C3aR and C5aR complement receptors, respectively. Activation through any of these receptors leads to histamine release, eicosanoid synthesis, IL-4 and IL-13 gene expression. Priming is the capacity for molecules that cannot maximally

activate basophils on their own to augment FcεRI-mediated activation (21). The mechanisms that regulate basophil releasability are not entirely known. Cytokines and growth factors such as IL-3, but also IL-1, IL-5, GM-CSF, stem-cell factor (SCF), nerve growth factor (NGF), histamine releasing factor (HRF), and IFN-γ are capable of potentiating or priming histamine and LTC_4 (Figure 3) (11, 12, 13, 14, 21, 53, 72, 74, 97). The presence of such mediators at sites of allergen exposure may lower the threshold for the development of allergic inflammation.

Figure 2. Human FcεRIα molecule. The overall structure of the FcεRIα molecule is an inverted V shape. The binding site for IgE is found at the top of the molecule, and the cell membrane at the bottom (modified after reference 43).

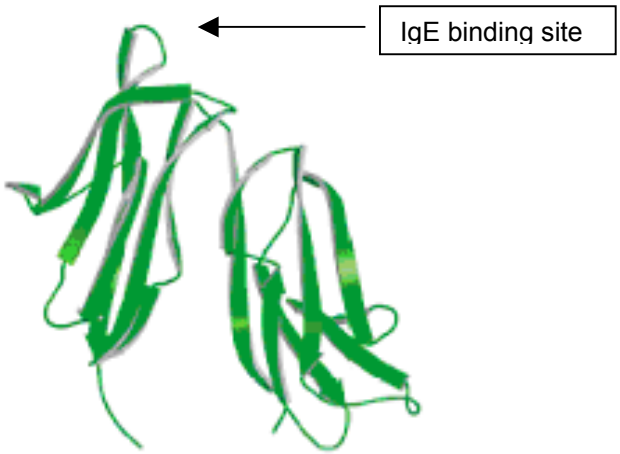
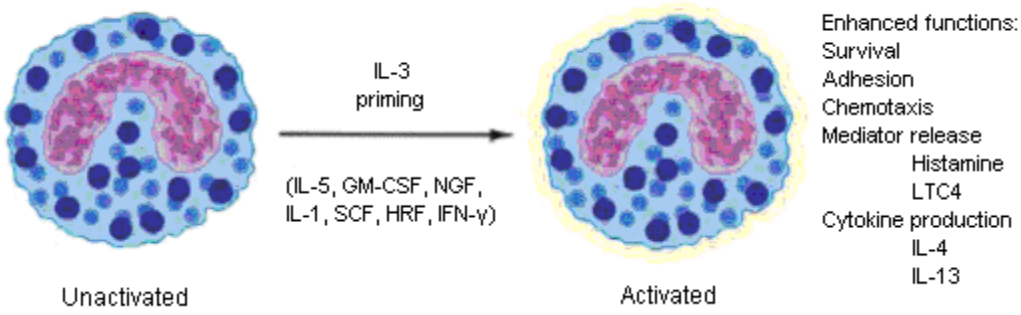


Figure 3. Examples of functional changes associated with basophil priming and activation. IL-3 is considered the most effective amd specific cytokine for inducing functional changes, although the other cytokines listed are also capable of inducing many, if not all, of these changes (21).



1.1.4. Mediators

Basophils produce many mediators, similar to mast cells, such as histamine, leukotrienes, IL-4, and IL-13 (100). Conversely, the mast-cell mediators PGD_2 and IL-5 are not produced by basophils. Of the newly synthesized eicosanoid mediators, basophils primarily generate LTC_4. In addition to histamine, basophil granules contain a number of other preformed mediators, such as chondroitin sulfate, major basic protein, and Charcot-Leyden crystal protein. Typically, basophils contain only small amounts of tryptase; however, there appears to be a great deal of variation among individuals with regard to basophil tryptase expression (39, 67).

In addition to their role in immediate hypersensivity, basophils may contribute to allergic inflammation through a number of nonclassic mechanisms. Basophil expression of IL-4 and CD40L induces B-cell IgE switching *in vitro* and may comprise an alternative mechanism promoting IgE class switching. Alternatively, the rapid and abundant expression of IL-4 by basophils has been suggested as a source of IL-4, which could further drive TH_2 cell differentiation.

1.1.5. Roles in health and disease

Although mast cells and basophils were identified over 100 years ago, their physiologic role in the body has remained a mystery. Both cells possess high affinity receptors for IgE on their surface membranes and are perhaps best known for mediating the pathophysiology of allergic diseases (37, 62). Type I of the Gell-Coombs classification (45, 93) are reactions in which antigens (allergens) combine with specific IgE antibodies that are bound to membrane receptors on mast cells and

basophils (Figures 4, 5 and 6). The antigen-antibody reaction causes the rapid release of their mediators. The mediators produce vasodilatation, increased capillary permeability, glandular hypersecretion, smooth muscle spasm, and tissue infiltration with eosinophils and other inflammatory cells (8).

Increased numbers of basophils in the affected tissue following experimental allergen challenge have now been reported in several studies. Basophils have been demostrated in lung and sputum of allergic asthmatics, in nasal mucosa and secretion of allergic rhinitis patients, and in skin lesions of atopic dermatitis patients. The number of basophils correlates with the severity of the disease (62). In a recent study (115) basophils were enumerated in nasal tissue collected from subjects with seasonal allergic rhinitis outside the pollen season and from the the same individuals two years later at the height of the pollen season. Over this period, subjects received a course of allergen-specific immunotherapy or placebo. In untreated subjects, basophils as detected immunohistochemically with 2D7 antibody were found to be numerous in both the submucosa and epithelium in tissues collected during the pollen season. In keeping with previous studies, seasonal increases in numbers of eosinophils and mast cells, but not neutrophils, were noted in biopsy tissues. Basophil infiltration into the epithelium during the pollen season appeared to be reduced by allergen-specific immunotherapy, an observation made also for eosinophils. This study thus provides further evidence for a role for basophils in the pathogenesis of seasonal allergic rhinitis, and the authors suggest that immunotherapy may work, at least in part, by reducing the recruitment of basophils to the nasal tissues.

The precise role of basophils in asthma pathogenesis is unclear. After allergen challenge, basophils are the predominant IL-4-expressing cell type in human asthmatic airways (85), peripheral blood mononuclear cells (32), and a mouse model

of asthma (69). Recent evidence has implicated the pleiotropic cytokine IL-13, which is produced in large amounts by activated basophils, as a key mediator of allergic asthma (64, 113). By means of basophil-specific monoclonal antibodies, basophils have been identified in cutaneous (44) and pulmonary (71) late-phase responses and are found in increased numbers in the lungs after fatal asthma episodes (58).

Considering that mast cells and basophils have been preserved through evolution, they must serve a valuable function in the body. Since the most common cause of mortality and morbidity in humans and animals are infectious diseases a compelling avenue to investigate mast cell and basophil function is in the contest of microbial infection. Intrinsically, mast cells and basophils are ideally placed and well endowed with inflammatory mediators to play a critical role in immune surveillance. Basophils are found in the circulatory system which ensures early contact with pathogens either because the circulatory system is a common conduit for the dissemination of microorganisms within the host or because it is a convenient site from where basophils can readily converge on distal extravascular sites of microbial attack. Based on their active role in allergic disease, it is clear that both mast cells and basophils have the innate capacity to mobilise a rapid and vigorous inflammatory response in the host.

Basophils and mast cells have been proposed to play an important role in the innate immune response (1). The current evidence for the role of basophils is circumstantial. Such a role would require the ability of basophils to be activated in a non-antigen-specific manner. These cells also recognize microbes in the absence of IgE as it can be deduced from early studies reporting direct basophil activation by various bacteria (28). Furthermore, other data support that although lipopolysaccharide (LPS), the major structural component of the outer wall of Gram-negative bacteria, did not induce mediator release in basophils *per se*, they potentiated basophil response to

other agonists (83). Lipopolysaccharide has also been reported to induce histamine release from basophils through complement activation (84).

Protein Fv, a protein that is synthesized by the liver, Protein L and A from *Peptostreptococcus magnus* and *Staphylococcus aureus*, respectively and gp120 from HIV-1, can activate human FcεRI+ cells to release proinflammatory mediators and cytokines *in vitro*, and in some settings *in vivo* (46, 47, 73).

Taken together, the observation that different classes of molecules derived from pathogens or endogenous sources can directly activate basophils independently of the presence of specific IgE on their surface, fulfills the theoretical requirement for a role of this cell type in innate immunity or the bridging of innate and specific immunity by skewing the differentiation of naive T cells to the TH_2 phenotype.

Mast cells and basophils contribute to certain aspects of the adaptive immune response to pathogens, especially their IgE-mediated responses to certain parasitic infections (38, 66, 78). This proposed role in parasite host defence is further suggested by the recent discovery of functional parasite homologs of histamine-releasing factor in the translationally controlled tumor protein family (70, 89).

Figure 4. Three-dimensional structure of grass allergen Phl P2 (119).

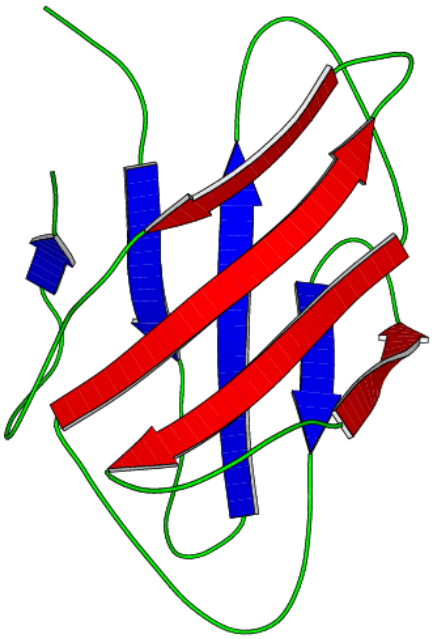


Figure 5. Schematic structure of Immunoglobulin E (120).

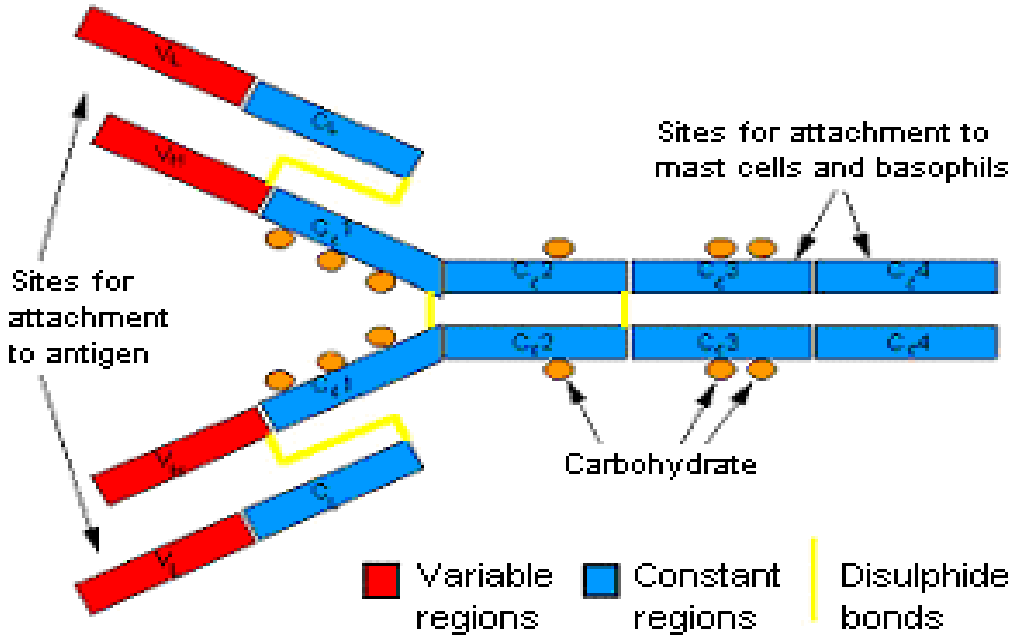
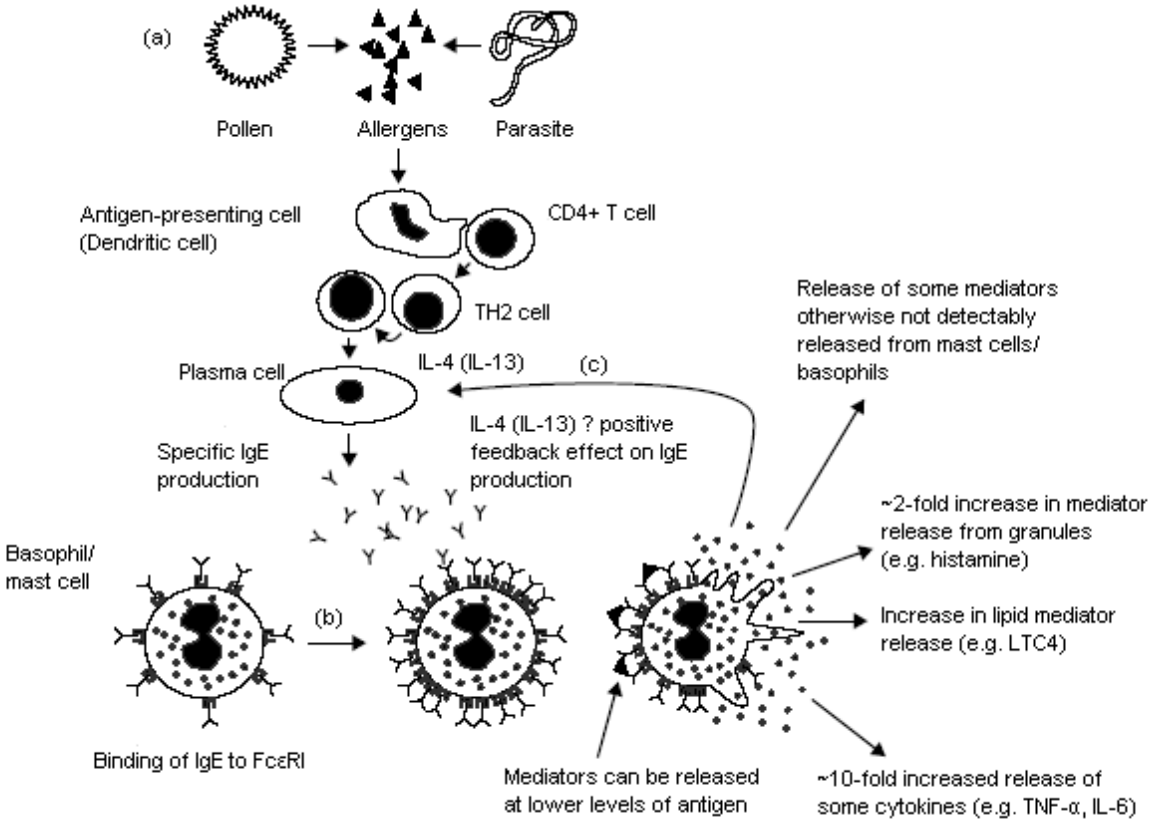


Figure 6. The mechanism of Typ I hypersensitivity reactions. Enhanced IgE-dependent effector function and potential immunoregulatory function in mast cells or basophils after IgE-dependent upregulation of FcεRI surface expression. **(a)** Various factors can trigger immune responses whereby specific IgE is produced by plasma cells. **(b)** Binding of IgE upregulates its receptors and results in response at lower levels of antigen (e.g. allergens) and in increased release of mediators and cytokines. **(c)** There may also be a positive feedback via IL-4 and IL-13 that increases IgE production (112).



1.2. The immune system

We are continually exposed to microorganisms that are inhaled, swallowed, or inhabit our skin and mucous membranes. Whether these microorganisms penetrate and cause disease is a result of both the pathogenicity of the microorganism (the virulence factors at its disposal) and the integrity of host defence mechanisms. The immune system is an interactive network of lymphoid organs, cells, humoral factors, and cytokines. The essential function of the immune system in host defence is best illustrated when it goes wrong; underactivity resulting in the severe infections and tumours of immunodeficiency, overactivity in allergic and autoimmune disease.

Immunity is divided into two parts determined by the speed and specificity of the reaction. These are named the innate and the adaptive responses, although in practice there is much interaction between them (86).

1.2.1. Innate immune response

Innate immune responses are characterized by the lack of immunologic memory. These immune reactions are less complicated than adaptive responses and therefore developed earlier in evolution (75). Nevertheless, failures in these `primitive` immune responses may be associated with severe, even fatal health problems. The innate response is rapid and less controlled than the adaptive immune response. Essential components of the innate response are neutrophils, eosinophils, natural killer cells, mast cells, basophils, cytokines, complement, antimicrobial peptides (101) and dendritic cells, positioned at the interface of innate and adaptive immunity (7, 55).

1.2.2. Adaptive immune response

The characteristic features of an adaptive immune response are its specificity and its improvement with each successive encounter with the same antigen due to the accumulation of a form of memory (30, 86). A crucial event during the generation of an adaptive immune response is antigen presentation. Adaptive immunity is the hallmark of the immune system of higher animals. This response consists of antigen-specific reactions through T and B lymphocytes.

1.2.3. Toll-like receptors

Innate immunity recognizes invading microorganisms and induces a host defense response. The molecular mechanisms that underlie innate immune recognition remained quite unclear until recently. Studies of the host defence system in fruit flies provided the first clue as to the mechanism of innate immune recognition. In *Drosophila*, a family of Toll receptors plays an important role in combating the invasion of pathogens (52). Subsequently, homologues of *Drosophila* Toll were identified in mammals, and are now termed Toll-like receptors (TLRs) (76, 103).

Although currently eleven TLRs have been recognized, their might not be limited yet (55, 117). They are selectively expressed among immune cells, where they induced a variety of responses. Each TLR recognizes a restricted subset or even a single molecule produced by microbes (Figure 7, Table 1).

TLR2 is essential for the recognition of lipoproteins and peptidoglycans and TLR3 recognizes a viral double-stranded RNA and polyinosine-polycytidylic acid [poly(I:C)], a synthetic molecule resembling double-stranded RNA. TLR4 recognizes

lipopolysaccharide (LPS), and TLR5 recognizes flagellin, a component of bacterial flagella. TLR6 participates in the discrimination of lipoproteins, TLR7 recognizes small synthetic immune modifiers including imiquimod and TLR9 recognizes unmethylated CpG motifs frequently found in the genome of bacteria and viruses, but not vertebrates. Another open question is whether or not abnormal TLR activation by endogenous ligands is involved in immunological disorders such as autoimmune diseases and chronic inflammatory responses.

Toll-like receptor signaling pathways originate from the cytoplasmic Toll/IL-1 receptor (TIR) domain. A TIR domain-containing adaptor, MyD88, associates with the cytoplasmic TIR domain of TLRs, and recruits IRAK to the receptor upon ligand binding. IRAK then activates TRAF6, leading to the activation of the IκB kinase (IKK) complex consisting of IKKα, IKKβ and NEMO/IKKγ. The IKK complex phosphorylates IκB, resulting in nuclear translocation of NFκB which induces expression of inflammatory cytokines. TIRAP, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signaling pathway via TLR2 and TLR4. In TLR3- and TLR4-mediated signaling pathways, activation of IRF-3 and induction of IFN-β are observed in a MyD88-independent manner. A third TIR domain-containing adaptor, TRIF, is essential for the MyD88-independent pathway. Non-typical IKKs, IKKi/IKKε and TBK1, mediate activation of IRF-3 downstream of TRIF. A fourth TIR domain-containing adaptor, TRAM, is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway (Figure 8) (9, 104).

Toll-like receptor family members are expressed differentially among immune cells (81). Surface expression of TLRs, as measured by monoclonal antibody (mAb) binding, seems to be very low. It corresponds to a few thousand molecules per cell in monocytes and a few hundred or less in immature dendritic cells (110). However, TLR expression is observed in a variety of other cells, including neutrophils, mast

cells, basophils, keratinocytes (63), vascular endothelial cells, adipocytes, cardiac monocytes and intestinal cells.

Among the innate immune cells, immature dendritic cells, which are capable of taking up pathogens by various mechanisms (phagocytosis, pinocytosis etc.) express several kinds of TLRs. The immature dendritic cells mature after the recognition of microbial components via TLRs. The mature dendritic cells in turn present pathogen-derived antigen, express co-stimulatory molecules, secrete several inflammatory cytokines including IL-12, and interact with naive T cells. The naive T cells harbouring the antigen-specific T cell receptor are instructed to develop into TH_1 cells, and clonally expand to exhibit effective adaptive immune responses. Therefore, TLRs are regarded as molecules bridging the gap between innate and adaptive immunity (4, 103).

As originally proposed, the hygiene hypothesis suggested that infections induce TH_1-biased immunoresponsiveness, which in turn protects the host from diseases fueled by TH_2-biased immune dysregulation (102, 114). Until recently, most TLR/ligand interactions had been shown to favor TH_1-like responses rather than promoting TH_2 responses commonly associated with allergic disease. However, this concept was recently challenged in rodent models, in which both TLR ligand concentration and route of immunization were shown to play important roles in determining whether type 1 or type 2 immune responses develop (27, 36, 91).

Figure 7. Toll-like receptors and their ligands (modified after reference 104).

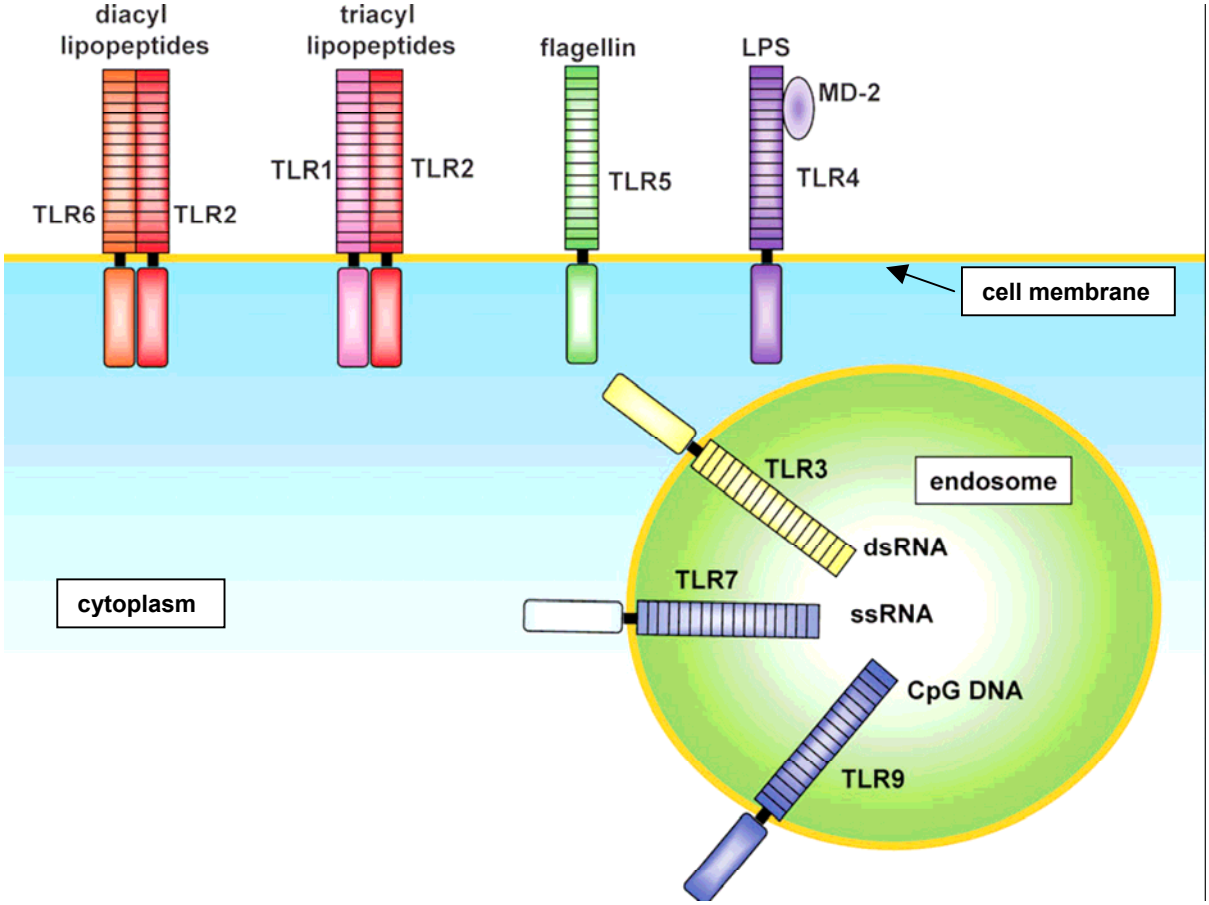
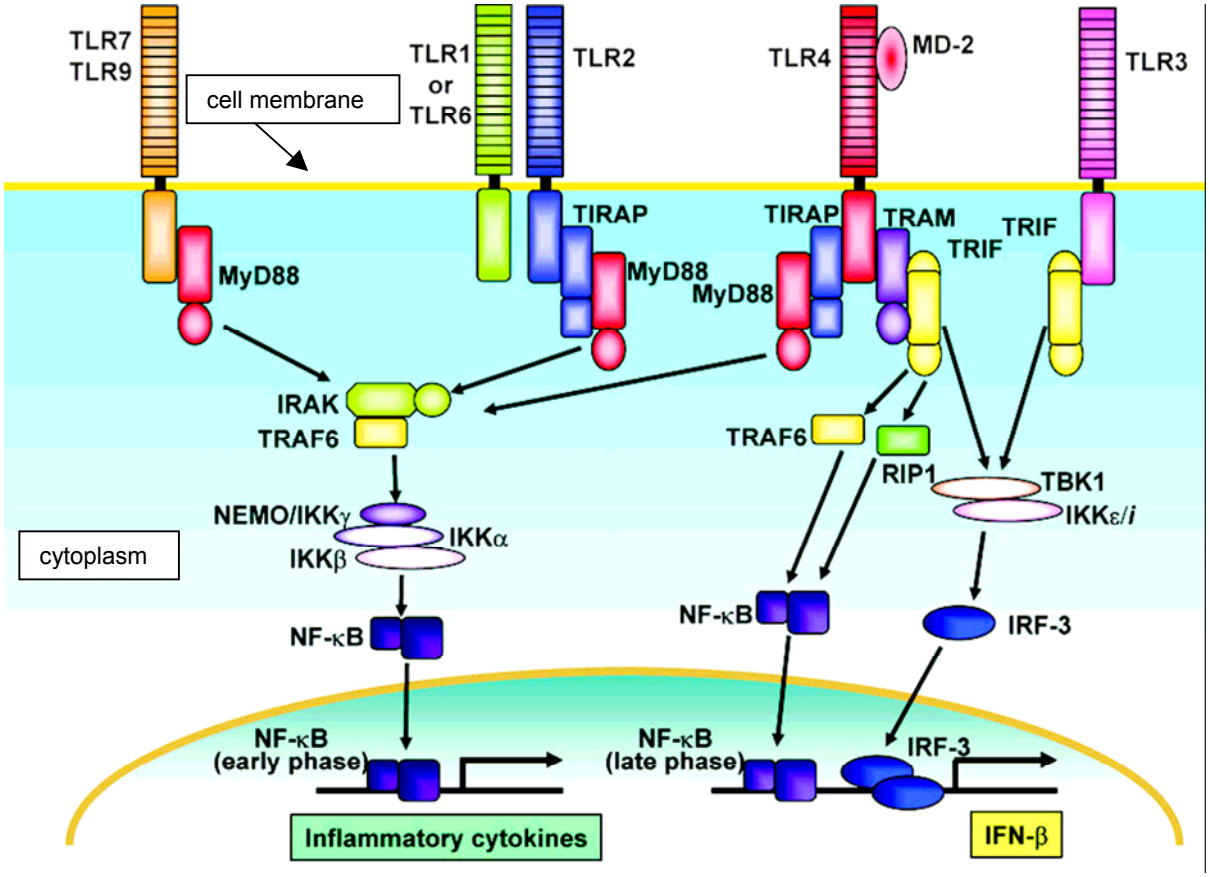


Table 1. Toll-like receptors and their ligands (3, 50, 80).

Toll-like receptor (TLR)	Identified ligands
TLR1/TLR2	Tri-acyl lipopeptides (bacterial, mycoplasmal, soluble factors)
TLR2	Peptidoglycan, lipopeptide (Pam_3Cys), zymosan, glycosylphosphoinositols, glycolipids, LTA, porins, atypical LPS, HSP70 (host)
TLR3	dsRNA (viral), poly(I:C) (synthetic)
TLR4	LPS, Taxol (plant), fusion and envelope proteins (viral), HSP60 (bacterial), multiple host proteins
TLR5	Flagellin
TLR6/TLR2	Di-acyl lipopeptide (mycoplasma)
TLR7	Synthetic ligands: Imidazoquinolines (Imiquimod and R-848), Loxoribine, Bropirimine, ssRNA
TLR8	ssRNA
TLR9	unmethylated CpG DNA, Chromatin-IgG complexes
TLR10	?
TLR11	Uropathogenic bacteria

Figure 8. Toll-like receptor signaling pathway (modified after reference 104).



1.2.4. Toll-like receptors and basophils

Human basophils were recently found to express high levels of TLR2 and TLR4 compared with other granulocytes (10, 94), and to possess mRNA for both TLR1 and TLR6 (55), so it is likely that these heterodimers are expressed on basophils.

These findings raise the possibility of TLR involvement in mediating basophil responses.

1.2.5. Ligand recognition by Toll-like receptors: lipopolysaccharide, polyinosine-polycytidylic acid and peptidoglycan

Lipopolysaccharide (LPS), the major structural component of the outer wall of Gram-negative bacteria, is a potent activator of the immune system. Structurally, LPS is a complex glycolipid composed of a hydrophilic polysaccharide portion and a hydrophobic domain known as lipid A. The shape of lipid A, the component responsible for the immunostimulatory activity of LPS, has been shown to direct the interaction of LPS with TLRs (82).

Large quantities of LPS induce the overproduction of cytokines causing septic shock while suboptimal doses of LPS induce tolerance to subsequent exposure to LPS (40). LPS recognition is predominantly mediated by TLR4 (87). This recognition involves the binding of LPS with lipopolysyccharide-binding protein (LBP) and subsequently with CD14 which physically associates with a complex including TLR4 and MD2 (90). Formation of the TLR4-centered LPS receptor complex induces the production of proinflammatory cytokines through the MyD88 pathway.

Lipopolysaccharide signaling also involves a MyD88-independent cascade that mediates the expression of IFN-inducible genes.

Polyinosine-polycytidylic acid [poly(I:C)] is a synthetic mimic of viral double-stranded RNA (dsRNA) that is thought to be an important viral pathogen-associated molecular pattern. Recently, TLR3 knockout mice have been generated and shown to have reduced responses to poly(I:C), suggesting that TLR3 is involved in recognition of dsRNA (5).

This may indicate the role of TLR3 in viral recognition. TLR3-mediated recognition of viruses or dsRNA results in TRIF-dependent activation of IRF-3 and NFκB. However, viruses or dsRNA are also recognized in a TLR3-independent manner, since the impairment of the responsiveness to viruses or dsRNA in TLR3-deficient mice is only partial. Retinoic acid-inducible gene I (RIG-I) was identified as another molecule that is responsible for viral recognition and that mediates activation of IRF-3 (Figure 9) (104, 116).

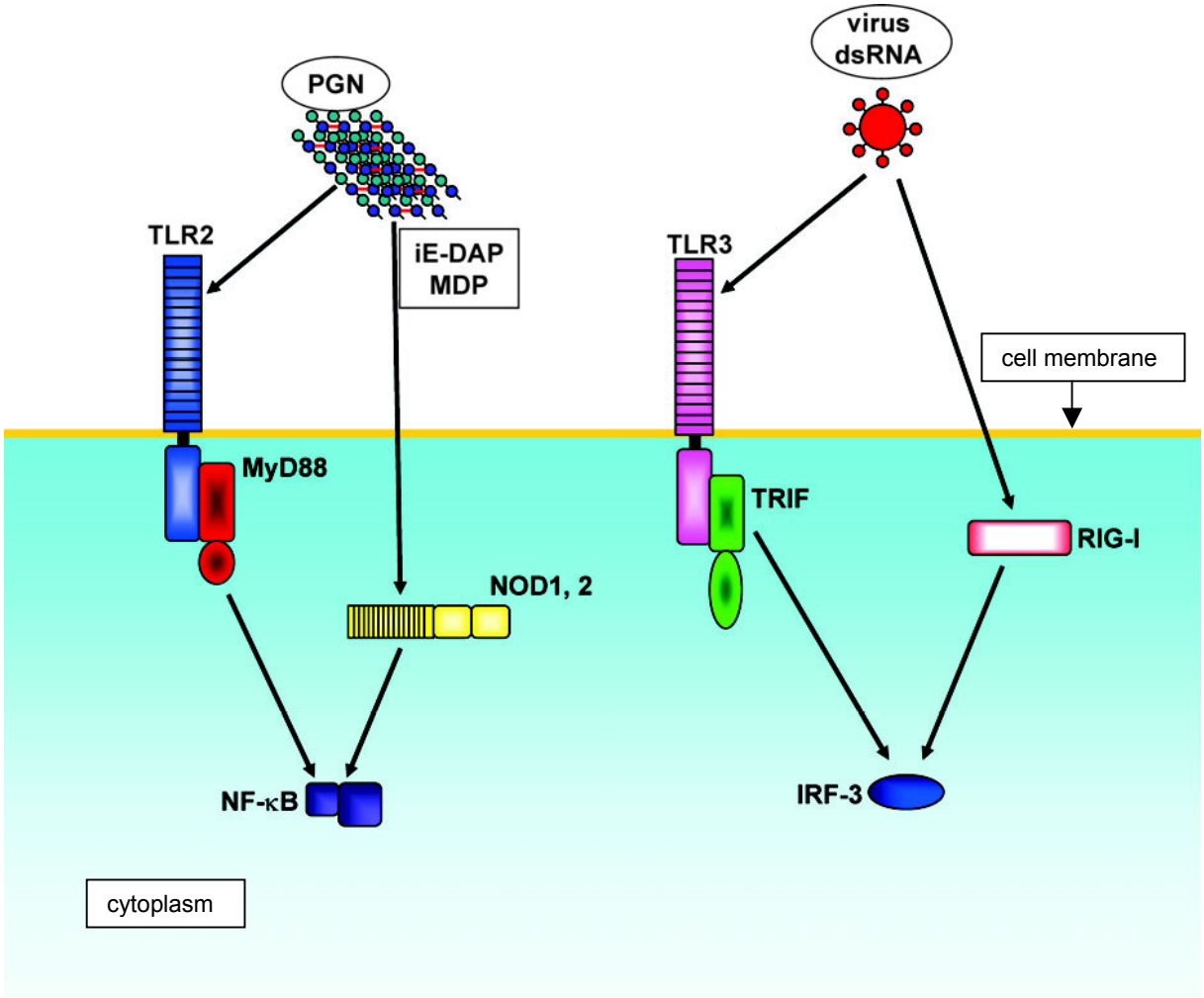
Peptidoglycan (PGN) has previously been shown to be recognized by TLR2 (105). However, PGN is a thick rigid layer that is composed of an overlapping lattice of two sugars that are crosslinked by amino acid bridges, and the exact structure of PGN that is recognized by TLR2 remains unclear. Recent accumulating evidence indicates that the nucleotide-binding oligomerization domain (NOD) family of proteins plays an important role in the recognition of intracellular bacteria (Figure 9).

NOD1 was originally identified as a molecule that is structurally related to the apoptosis regulator, Apaf-1. It contains a caspase-recruitment domain (CARD), a NOD domain and a C-terminal LRR domain. Recent studies have demonstrated that overexpression of NOD1 enables 293 cells to respond to preparations of PGN (26, 48). Characterization of the PGN motif detected by NOD1 revealed that γ-D-glutamyl-meso diaminopimelic acid (iE-DAP) is the minimal structure required for NOD1

detection. NOD2 was identified as a molecule that shows structural similarity to NOD1, but which possesses two CARD domains in its N-terminal region. Similar to NOD1, expression of NOD2 confers responsiveness to PGN in 293 cells. Biochemical analyses identified the essential structure recognized by NOD2 as muramyl dipeptide MurNAc-L-Ala-DisoGln (MDP) derived from PGN (49, 54). Thus, NOD1 and NOD2 recognize different structures within PGN. MDP is found in almost all bacteria, whereas iE-DAP is restricted to Gram-negative bacteria. Therefore, NOD1 may play an important role in sensing Gram-negative bacterial infection inside cells.

Although TLR2 has been reported to recognize PGN, it is possible that TLR2 recognizes lipoprotein/lipopeptide contaminants that are trapped within the layers of the PGN mesh (22, 104, 108).

Figure 9. TLR-dependent and –independent recognition of microbial components (modified after reference 104).



2. Objective

Basophils express a complete and functional FcεRI receptor ($\alpha\beta\gamma_2$), cross-linking of which leads to basophil activation. Activated basophils express on their surface CD63, a tetraspan antigen and produce many mediators, such as histamine and leukotrienes.

In this study, we investigated the effect of lipopolysaccharide (LPS), polyinosine-polycytidylic acid [poly(I:C)] and peptidoglycan (PGN) on basophils from subjects with allergy. More precisely, whether these TLR agonists enhanced CD63 expression of basophils in response to allergen (Timothy grass). Furthermore, we tested whether LPS, poly(I:C) and PGN augment sulfidoleukotriene (sLT) production from basophils stimulated with allergen.

3. Materials and methods

3.1. Materials

Table 2. Reagents for cell stimulation.

Reagent	Company
LPS	Invivogen, San Diego, CA, USA
Poly(I:C)	Sigma-Aldrich, St. Luis, MO, USA
PGN	Invivogen
Stimulation Buffer With heparin and IL-3	Bühlmann Laboratories, Schönenbuch, Switzerland
Stimulation Control Anti-FcεRI mAb	Bühlmann Laboratories
Allergen Timothy grass	Bühlmann Laboratories

Table 3. Reagents for flow cytometry.

Reagent	Company
Staining Reagent Mix of anti-CD63-PE and Anti-IgE-FITC mAb	Bühlmann Laboratories
Lysing Reagent	Bühlmann Laboratories
Blocking Buffer	Bühlmann Laboratories

Table 4. Reagents for ELISA.

Reagent	Company
Microtiter Plate precoated with rabbit anti-mouse IgG	Bühlmann Laboratories
Plate Sealer	Bühlmann Laboratories
Wash Buffer *Concentrate (20X)* With preservatives	Bühlmann Laboratories
ELISA Buffer With preservatives	Bühlmann Laboratories
Calibrator Leukotriene D_4 in a buffer matrix	Bühlmann Laboratories
Blanking Reagent Leukotriene D_4 in a buffer matrix	Bühlmann Laboratories
Enzyme Label Leukotriene D_4 conjugated to aPase with preservatives	Bühlmann Laboratories
Antibody Mouse anti-sLT $C_4/D_4/E_4$ Ab in a buffer matrix with preservatives	Bühlmann Laboratories
pNPP Substrate	Bühlmann Laboratories
Stop Solution 2 N NaOH	Bühlmann Laboratories

Table 5. Mediums.

Medium	Company
RPMI 1640 Medium + 25mM HEPES + L-Glutamine	Invitrogen, Karlsruhe, Germany
Aqua ad inectabilia	Delta Select, Pfullingen, Germany

Table 6. Machines.

Machine	Company
Centrifuge 4K15	Sigma Laboratory Centrifuges, Osterode am Harz, Germany
Water Bath SW5	Stuart Scientific, Redhill Surrey, UK
Flow Cytometer FACScan	Becton Dickinson, Franklin Lakes, NJ, USA
Computer Software: Cell Quest Version 3.0f	Apple Computer, Cupertino, CA, USA Becton Dickinson
Photometer DYNEX MRXII	DYNEX Technologies, Chantilly, VA, USA

Blood collection system S-Monovette® was from Sarstedt (Nümbrecht, Germany). Other materials such as pipettes and polystyrene round-bottom tubes were from Becton Dickinson Labware Europe (Le Pont De Claix, France).

3.2. Methods

3.2.1. Sampling of blood and preparation of basophils

Four donors with allergy to grass were recruited. All subjects had a history of seasonal hay fever during summer, a positive skin test reaction (wheal >5 mm) to *Phleum pratense* (Timothy grass pollen) as well as increased serum allergen-specific IgE for grass pollen (DPC Biermann, Bad Nauheim, Germany). Donors did not receive any drugs, especially antihistamines and oral glucocorticoids, at least 5 days before blood donation and had not an infection or an active chronic inflammation. The clinical characteristics of the donors studied are presented in Table 7.

Table 7. Clinical characteristics of the donors. Allergen-specific IgE for grass pollen (Class 0: negative, Class 6: strongly positive).

Donor	Sex	Age	Allergen	Specific IgE / Class
1	Male	32	Grass pollen	4
2	Male	35	Grass pollen	6
3	Female	44	Grass pollen	3
4	Male	32	Grass pollen	6

Nine ml blood sample of donors was anticoagulated (1.6 mg EDTA / ml blood). The anti-coagulated blood sample was mixed by inverting the venipuncture tube at least 5 times. The venipuncture tube was centrifuged for 5 minutes at 200 x g and 2-8°C.

After the centrifugation step, two phases could be observed from top to the bottom of the tube: i) plasma fraction containing basophil-enriched leukocytes and ii) erythrocyte fraction.

The plasma fraction with the leukocytes on the top of the erythrocytes was collected with a disposable plastic pipette and transfered into a fresh and pyrogen-free 5 ml polystyrene tube.

The plasma fraction with the leukocytes, contaminated with a low amount of erythrocytes, was centrifuged for 10 minutes at 500 x g and 2-8°C.

After this centrifugation step, three phases can be observed from top to the bottom of the tube: i) plasma fraction, ii) leukocyte fraction and iii) erythrocyte fraction.

Plasma fraction was removed and 3 ml of RPMI medium (with 25mM HEPES and L-Glutamine) was added.

This sample, containing basophil-enriched leukocytes, was equally (1.5 ml each) separeted in two pyrogen-free 5 ml polystyrene tubes: sample 1 and sample 2.

The purity of basophils ranged form 10 to 30%, with contaminating cells consisting mainly of lymphocytes and variable proportions of neutrophils.

3.2.2. Basophil stimulation

For *in vitro* stimulation assays of basophils the following TLR agonists were used at the following concentrations (63): Lipopolysaccharide (LPS) from *Salmonella minnesota* (Invivogen) 1 µg/ml, polyinosine-polycytidylic acid [poly(I:C)] (Sigma-Aldrich) 20 µg/ml and peptidoglycan (PGN) from *Staphylococcus aureus* (Invivogen) 10 µg/ml. Fifteen µl of a Toll-like receptor agonist was added in sample 2. Sample 1 served as control. Cells were incubated at 37°C for 30 minutes. After incubation, the samples were centrifuged for 10 minutes at 400 x g and 2-8°C. Stimulation buffer (250 ml in each tube) was added.

Allergen (Timothy grass) was diluted with stimulation buffer, in order to obtain different concentrations. Concentrations of allergen were decided according to donor´s sensitivity (serum allergen-specific IgE for grass pollen) (Figure 10 and Table 8).

Pyrogen-free polystyrene tubes suited for flow cytometry measurements were labeled and pipeted as follow:

Tube 1	50 µl of stimulation buffer	50 µl of patient´s cell suspension from Sample 1
Tube 2	50 µl of stimulation control	50 µl of patient´s cell suspension from Sample 1
Tube 3	50 µl of allergen in concentration no. 1 (C1)	50 µl of patient´s cell suspension from Sample 1
Tube 4	50 µl of allergen in concentration no. 2 (C2)	50 µl of patient´s cell suspension from Sample 1

Tube 5	50 µl of allergen in concentration no. 3 (C3)	50 µl of patient´s cell suspension from Sample 1
Tube 6	50 µl of stimulation buffer	50 µl of patient´s cell suspension from Sample 2
Tube 7	50 µl of stimulation control	50 µl of patient´s cell suspension from Sample 2
Tube 8	50 µl of allergen in C1	50 µl of patient´s cell suspension from Sample 2
Tube 9	50 µl of allergen in C2	50 µl of patient´s cell suspension from Sample 2
Tube 10	50 µl of allergen in C3	50 µl of patient´s cell suspension from Sample 2

Tubes were vortexed gently, covered and incubated for 40 minutes at 37°C in a water bath. Fifty µl of cold blocking buffer was added in each tube and centrifuged for 5 minutes at 500 x g and 2-8°C. Supernatants were collected for sulfidoleukotrienes estimation by enzyme-linked immunosorbent assays (ELISAs) and the collected samples were stored at -27°C.

Figure 10. A representative experiment: basophil activation after incubation with various allergen concentrations. Donor 3 had allergen-specific IgE for grass pollen class 3. Basophil activation was very low at lower allergen concentrations, such as 0.5 ng/ml and 0.33 ng/ml. Arrows indicate the selected concentrations for further investigations with Donor 3.

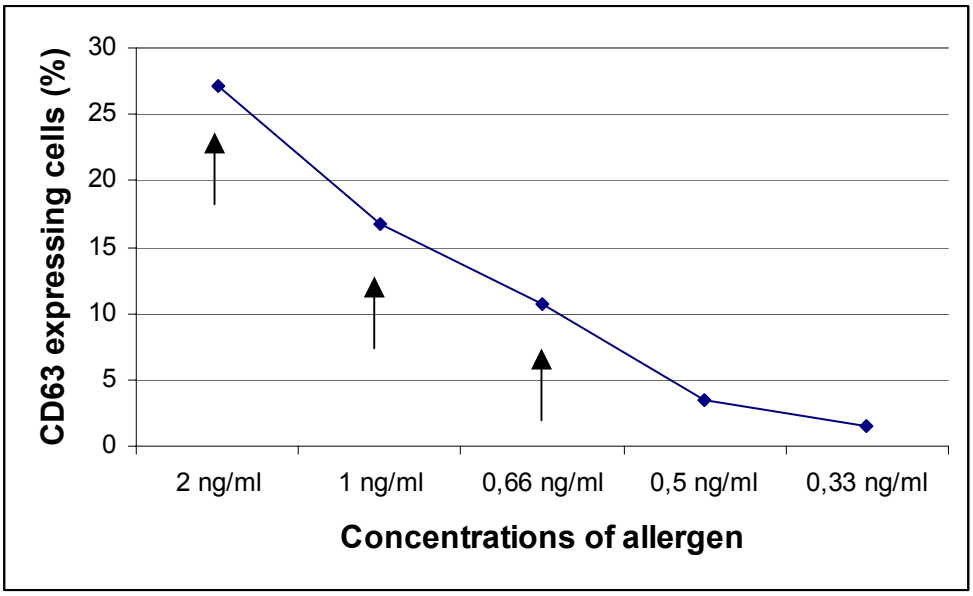


Table 8. Concentrations of allergen. Allergen (Timothy grass) diluted with stimulation buffer, in order to obtain different concentrations. Concentrations of allergen were decided according to donor´s sensitivity. Three concentrations (C1, C2, C3) were used for each of the donors.

Donor	C1	C2	C3
1	1 ng/ml	0.66 ng/ml	0.5 ng/ml
2	0.5 ng/ml	0.33 ng/ml	0.2 ng/ml
3	2 ng/ml	1 ng/ml	0.66 ng/ml
4	0.5 ng/ml	0.2 ng/ml	0.14 ng/ml

3.2.3. Cell staining

The cell pellet was re-suspended with 100 µl cold blocking buffer and 20 µl of cold staining reagent was added to each tube and vortexed gently. After staining, incubation followed in a dark room for 30 minutes at 2-8°C. The sedimented cells were vortexed gently and 2 ml of pre-warmed (18-28°C) lysing reagent was added to each tube and incubated in a dark room for 5 minutes at 18-28°C. One ml of blocking buffer was added to stop the reaction and centrifugation for 5 minutes at 100 x g at 2-8°C was followed. The supernatant was decanted, the cell pellet was re-suspended with 500 µl of blocking buffer and vortexed gently.

Analysis by flow cytometry was proceeded within 2 hours.

3.2.4. Flow cytometric analysis

Surface expression of various markers was assesed using CellQuest analysis software on a FACScan (Becton Dickinson) flow cytometer.

The analysis was based on three steps (Figure 11):

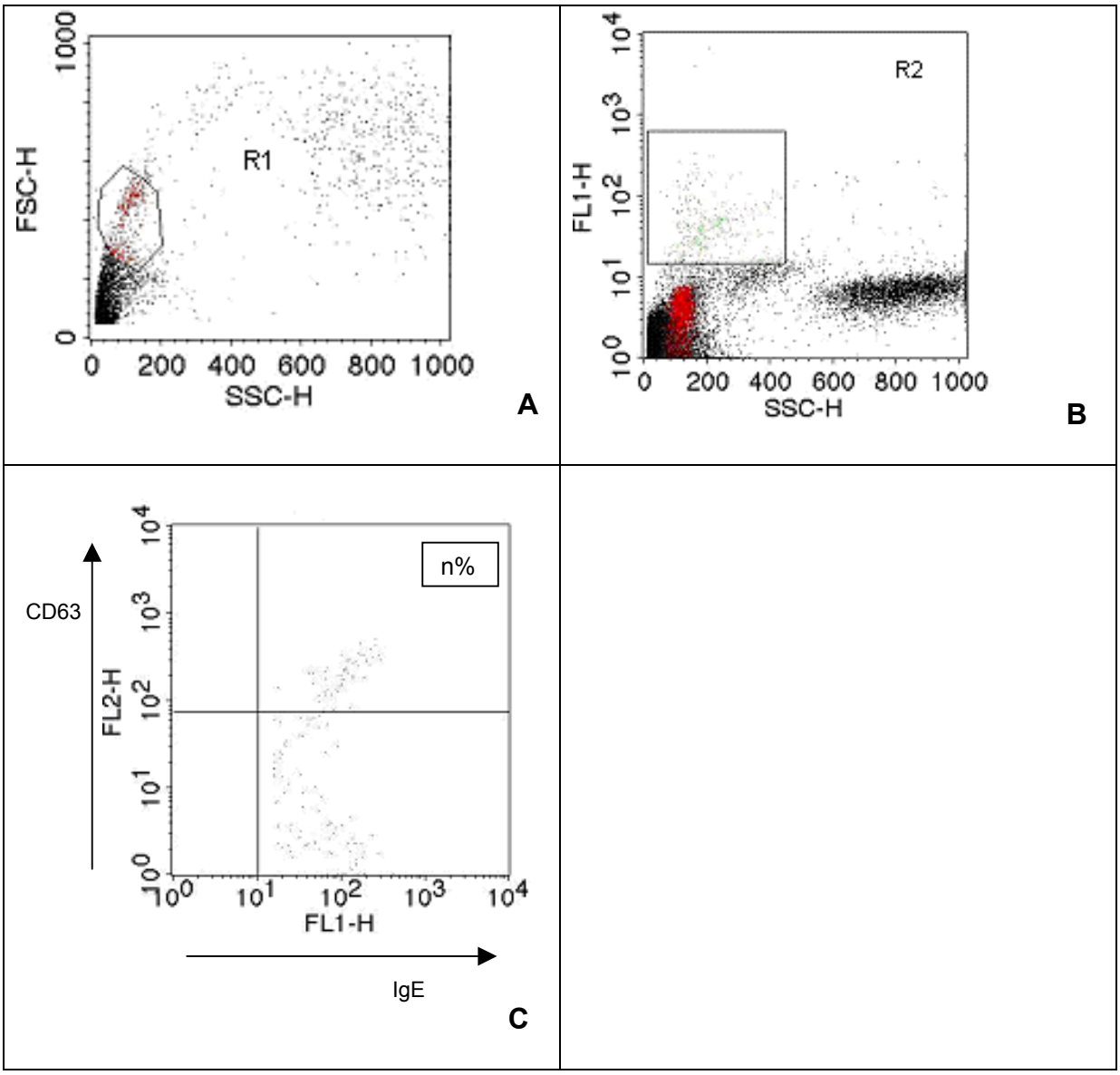
1. A gate 1 (R1) was set including the entire lymphocyte population.

2. We set within the lymphocyte population gate (R2) the brightly fluorescent FITC, IgE positive, cells.

3. The percentage of brightly fluorescent PE cells (CD63 positive cells) compared to the total amount of brightly fluorescent FITC cells was calculated by the cytometer software.

Typically, 20,000 to 450,000 total events were acquired to obtain adequate numbers of basophils (2000-5000).

Figure 11. Flow cytometric analysis. R1: a gate including the entire lymphocyte population (A). R2: a gate within the lymphocyte population, the brightly fluorescent FITC, IgE positive, cells (B). Percentage of brightly fluorescent PE cells (CD63 positive cells) compared to the total amount of brightly fluorescent FITC cells (C).



3.2.5. ELISA procedure

The ELISA (Enzyme Linked ImmunoSorbent Assay) is intended for the quantitative determination of sulfidoleukotrienes (sLT) produced by basophils upon contact with allergen alone and upon contact with allergen and TLR ligands.

We determinated the number of capture antibody-coated microtiter plate strips required to test plus 16 wells needed for running blanking reagent, calibrator and controls. The coated wells filled with storage buffer was emptied and washed once using 300 µl of wash buffer per well. The wells were emptied and the plate was stroken firmly onto blotting paper.

The following steps were subsequently performed:

- 100 µl of blanking reagent was pipeted in duplicate into wells A1 and A2.
- 100 µl of ELISA buffer (Zero Calibrator, S0) was pipeted in duplicate into wells B1 and B2.
- 100 µl of Calibrator S4 (50 pg/ml) was pipeted in duplicate into wells C1 and C2.
- 100 µl of Calibrator S3 (200 pg/ml) was pipeted in duplicate into wells D1 and D2.
- 100 µl of Calibrator S2 (800 pg/ml) was pipeted in duplicate into wells E1 and E2.
- 100 µl of Calibrator S1 (3200 pg/ml) was pipeted in duplicate into wells F1 and F2.
- 100 µl of each cell supernatant was pipeted into the subsequent wells.
- 50 µl of enzyme label and 50 µl of antibody were added to all wells.
- The plate was covered with a plate sealer, and incubated for 16-20 hours at 2-8°C, then removed, and the wells emptied and washed three times using 300 µl of wash buffer per well. The plate was stroken firmly onto blotting paper.
- 200 µl of pNPP substrate solution was added to all wells.
- The plate was covered with a plate sealer, and placed on a plate rotator set at 800-1000 rpm, protected from direct light and incubated for 30 minutes at 18-28°C.

- The plate sealer was removed and the reaction stopped by adding 50 µl of stop solution to all wells and mixed shortly on the microtiter plate rotator.
- The absorbance was read at 405 nm in a microtiter plate reader within 30 minutes.

3.2.6. Statistics

Comparison of two groups, named cells with or without TLR ligand, was determined using Student´s t-test. Statistical analysis was performed with Microsoft Excel for Windows Office XP. A P-value of < 0.05 was regarded as significant.

4. Results

4.1. CD63 expression on basophil surface

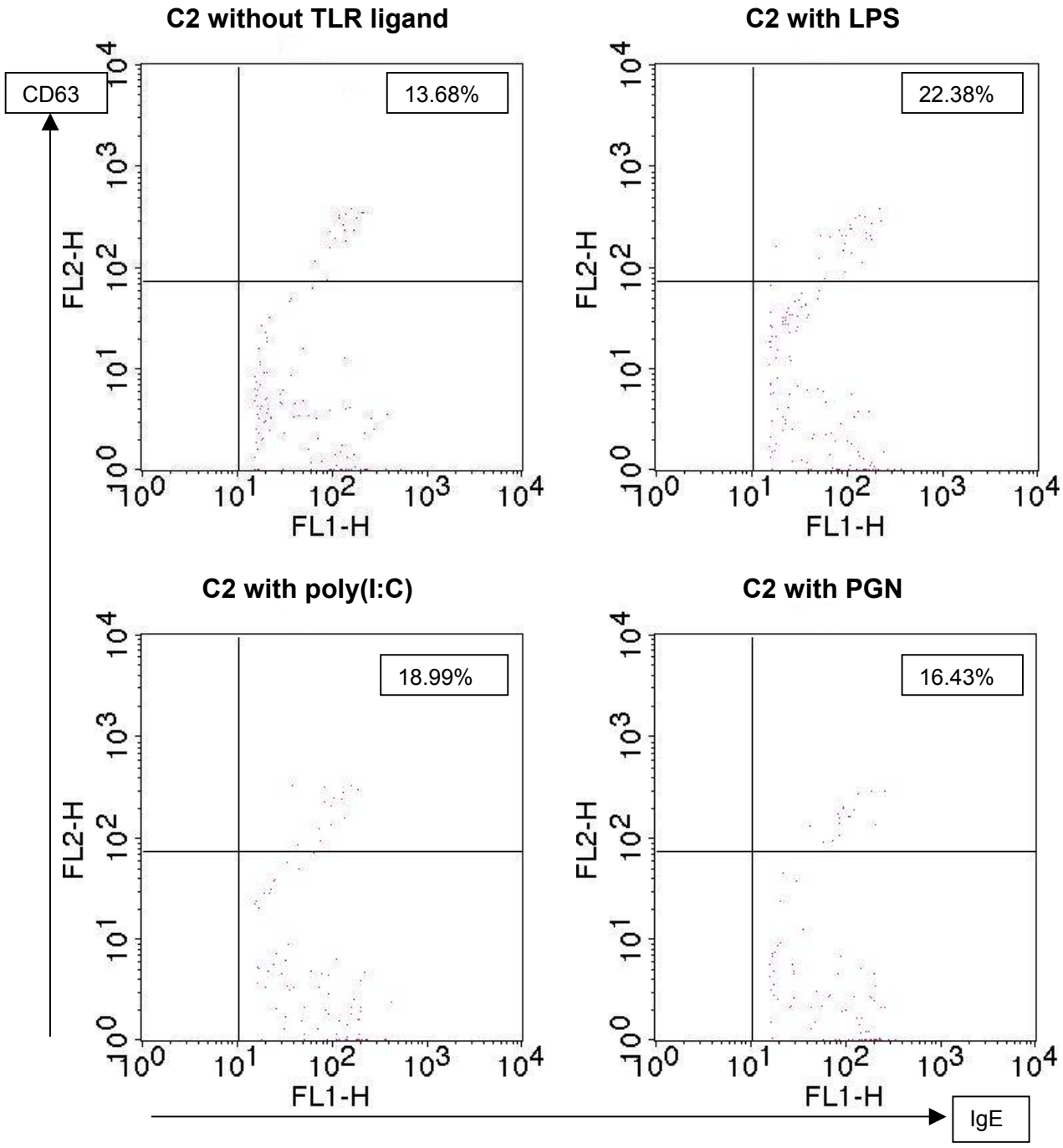
CD63 is a tetraspan antigen, a surface marker that appears *de novo* on the basophil surface during activation. The expression of CD63 appears to correlate with degranulation (61, 79). When exposed to an allergen or anti-IgE antibody, basophils express substantial amounts of CD63 on their surface.

We investigated CD63 expression (percentage) on the basophil surface after stimulation with various TLR ligands [LPS, poly(I:C) and PGN] and allergen (Timothy grass) in three concentrations (C1, C2, C3: higher, medium and lower allergen concentration respectively), for each donor as mentioned in Materials and Methods. We also calculated the arithmetic mean of CD63 expression of independent experiments with three donors tested with the same allergen concentration.

Two groups of cells were always compared: group of cells treated with allergen and group of cells treated with allergen and a TLR ligand. Differences between these two groups should have been higher than 3%, otherwise they were regarded as similar results.

Four donors with allergy to grass pollen were recruited and several tests for each donor and each TLR ligand were made. Basophil CD63 surface expression was measured using flow cytometry (Figure 12).

Figure 12. Flow cytometry. Representative example of results obtained by Donor 1 in C2 allergen concentration: without and with each of the three TLR ligands. The percentage of brightly fluorescent CD63 positive cells compared to the total amount of brightly fluorescent IgE positive cells was calculated.



4.1.1. Basophil activation after incubation with LPS and allergen

In all four donors treatment of basophils with heparin and IL-3 (negative control) alone or in combination with LPS did not induce CD63 surface expression. In Donor (D) 1 and D2 treatment of cells with LPS and anti-FcεRI mAb resulted in less CD63 surface expression compared with cells treated with anti-FcεRI mAb (positive control) alone. In D3 the expression was higher in the group of cells incubated with LPS and anti-FcεRI mAb, and in D4 the activation between the two groups was similar. However, in all four donors no significance level was reached.

In D1 the percentage of CD63-expressing cells was significantly higher in cells incubated with LPS and allergen than in cells incubated with allergen alone. Similar results to D1 was shown also in D2, D3 and D4, however, the level of significance was not always reached (Figure 13, Figure 14 and Table 9).

Basophils from D1, D2 and D4 treated with 0.5 ng/ml allergen concentration showed significantly higher CD63 basophil activation when preincubated with LPS (Figure 15).

Figure 13. Basophil activation after incubation with LPS and allergen (Donor 1 and 2). Basophil activation determined by percentage of surface CD63-expressing basophils. Columns show the arithmetic mean ± standard deviation of at least 3 independent experiments. N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.

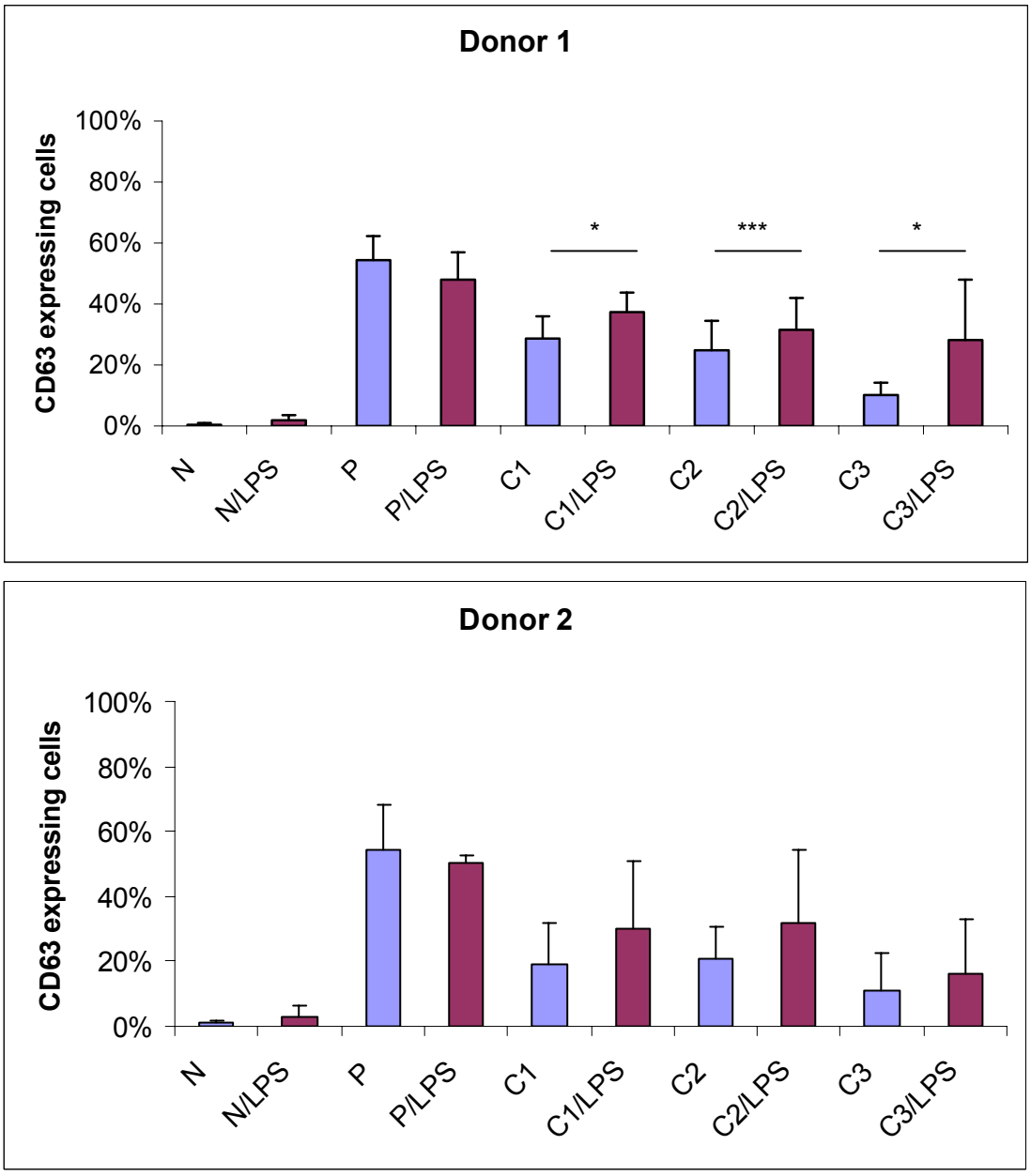


Figure 14. Basophil activation after incubation with LPS and allergen (Donor 3 and 4). Basophil activation determined by percentage of surface CD63-expressing basophils. Columns show the arithmetic mean ± standard deviation of at least 3 independent experiments. N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.

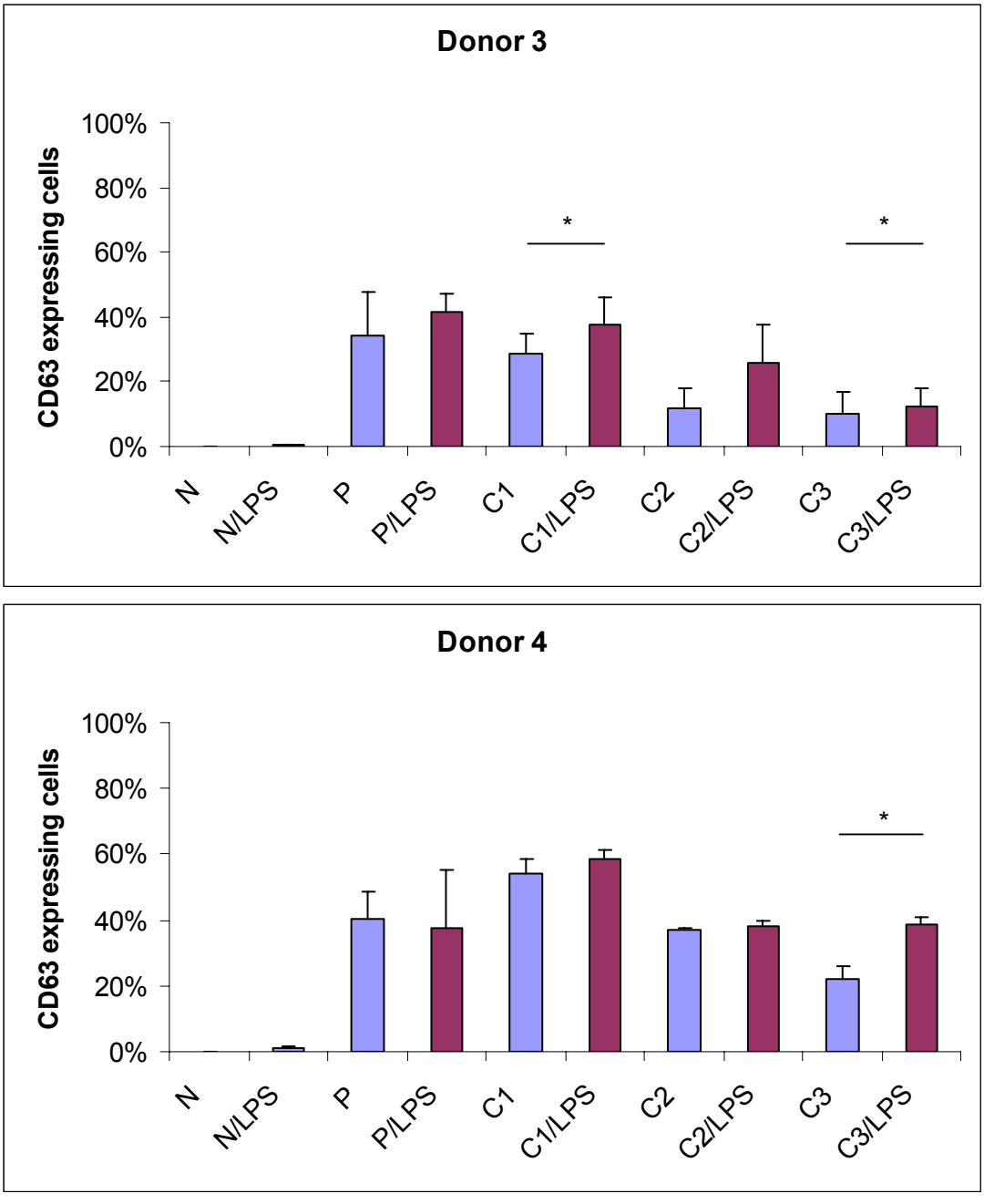
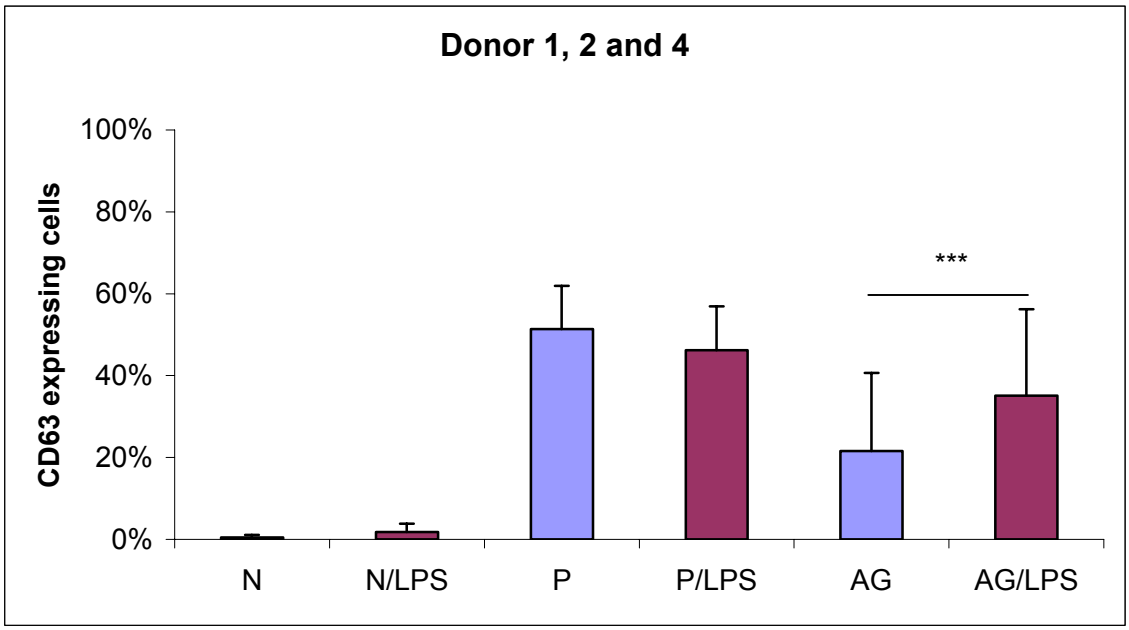


Table 9. Basophil activation after incubation with LPS and allergen. Basophil activation determined by percentage of surface CD63-expressing basophils (arithmetic mean of at least 3 independent experiments). N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Percent enhancement induced with LPS pretreatment. The level of significance is indicated by the P-value. N.s., not significant.

	without ligand (%)	**with ligand (%)**	**enhancement (%)**	**P**
Donor 1				
N	0.38	1.84	1.46	n.s.
P	54.43	47.98	-6.45	n.s.
C1	28.63	37.35	8,72	< 0.05
C2	24.82	31.52	6.7	< 0.001
C3	10.16	28.18	18.02	< 0.05
Donor 2				
N	1.14	2.70	1.56	n.s.
P	54.08	50.06	-4.02	n.s.
C1	19.13	29.86	10.73	n.s.
C2	20.53	31.76	11.23	n.s.
C3	10.86	16.31	5.45	n.s.
Donor 3				
N	0.15	0.29	0.14	n.s.
P	34.23	41.31	7.08	n.s.
C1	28.74	37.61	8.87	n.s.
C2	11.95	26.09	14.14	< 0.05
C3	10.23	12.45	2.22	< 0.05
Donor 4				
N	0.14	0.97	0.83	n.s.
P	40.47	37.63	-2.84	n.s.
C1	54.32	58.67	4.35	n.s.
C2	37.07	38.25	1.18	n.s.
C3	22.37	38.51	16.14	< 0.05

Figure 15. Basophil activation after incubation with LPS and allergen (Donor 1, 2 and 4). Basophil activation determined by percentage of surface CD63-expressing basophils. Columns show the arithmetic mean ± standard deviation of 14 independent experiments with the 3 donors. N: negative control, P: positive control, AG: allergen (0.5 ng/ml). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.



4.1.2. Basophil activation after incubation with poly(I:C) and allergen

In all four donors treatment of basophils with heparin and IL-3 (negative control) alone or in combination with poly(I:C) did not induce CD63 surface expression. In D1 and D4 treatment of cells with poly(I:C) and anti-FcεRI mAb resulted in less CD63 surface expression than incubation with anti-FcεRI mAb (positive control) alone; D2 and D3 showed similar results between the two groups. In all four donors, however, no significance level was reached.

In D1 basophils treated with allergen and poly(I:C) showed higher CD63 expression in all allergen concentrations (significantly higher in medium and lower allergen concentration) than cells treated with allergen alone. Similar results to D1 showed D2, however no significance level was reached. D3 showed similar results between the two groups in C1 and C3 allergen concentration, but higher basophil activation in the group of cells treated with TLR ligand and allergen in medium allergen concentration. In D4 the activation in cells treated with poly(I:C) and lower allergen concentration was significantly higher than allergen alone (Figure 16, Figure 17 and Table 10).

Basophils from D1, D2 and D4 treated with an allergen concentration of 0.5 ng/ml showed significantly higher CD63 basophil activation when preincubated with poly(I:C) (Figure 18).

Figure 16. Basophil activation after incubation with poly(I:C) and allergen (Donor 1 and 2). Basophil activation determined by percentage of surface CD63-expressing basophils. Columns show the arithmetic mean ± standard deviation of at least 3 independent experiments. N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.

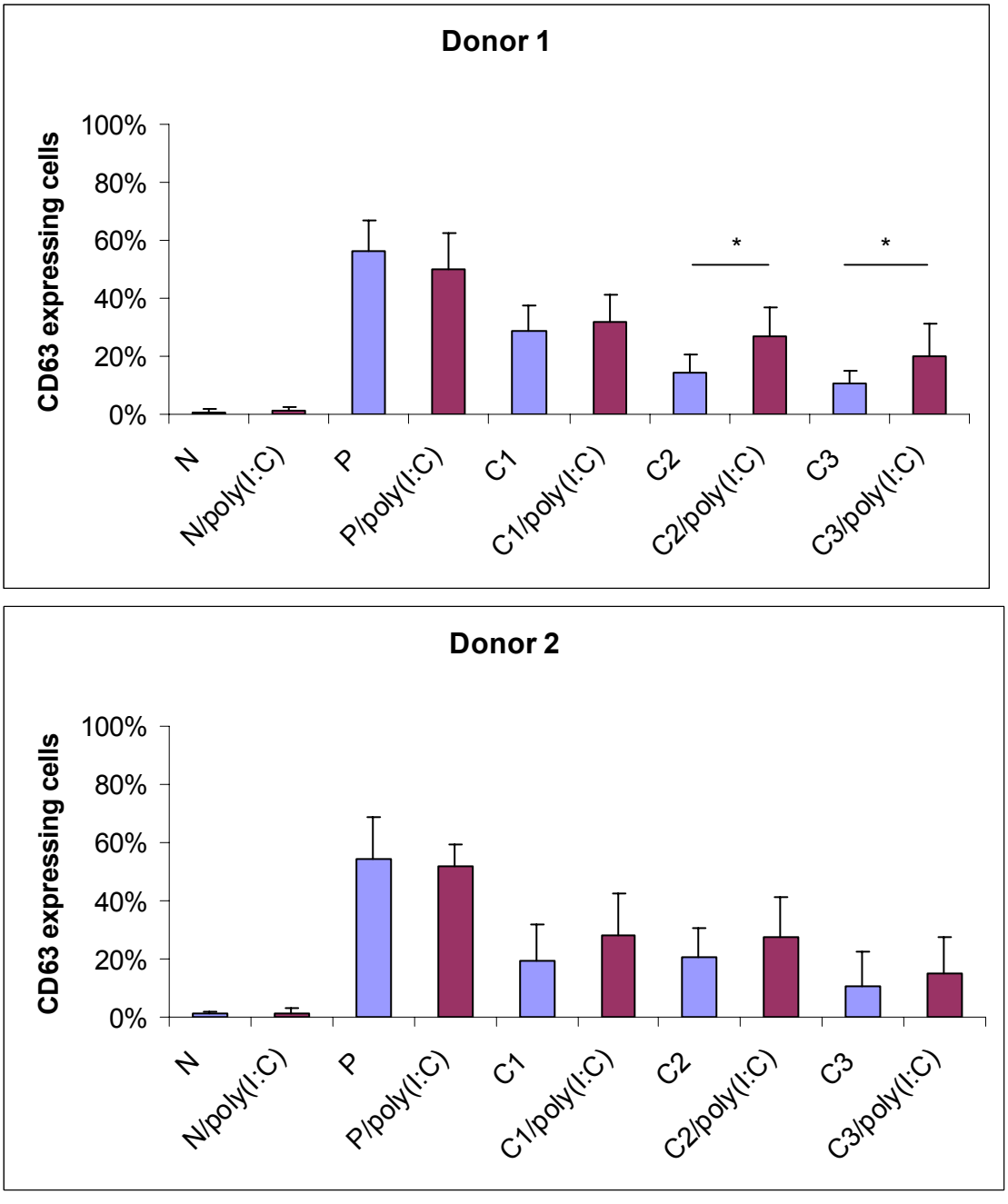


Figure 17. Basophil activation after incubation with poly(I:C) and allergen (Donor 3 and 4). Basophil activation determined by percentage of surface CD63-expressing basophils. Columns show the arithmetic mean ± standard deviation of at least 3 independent experiments. N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.

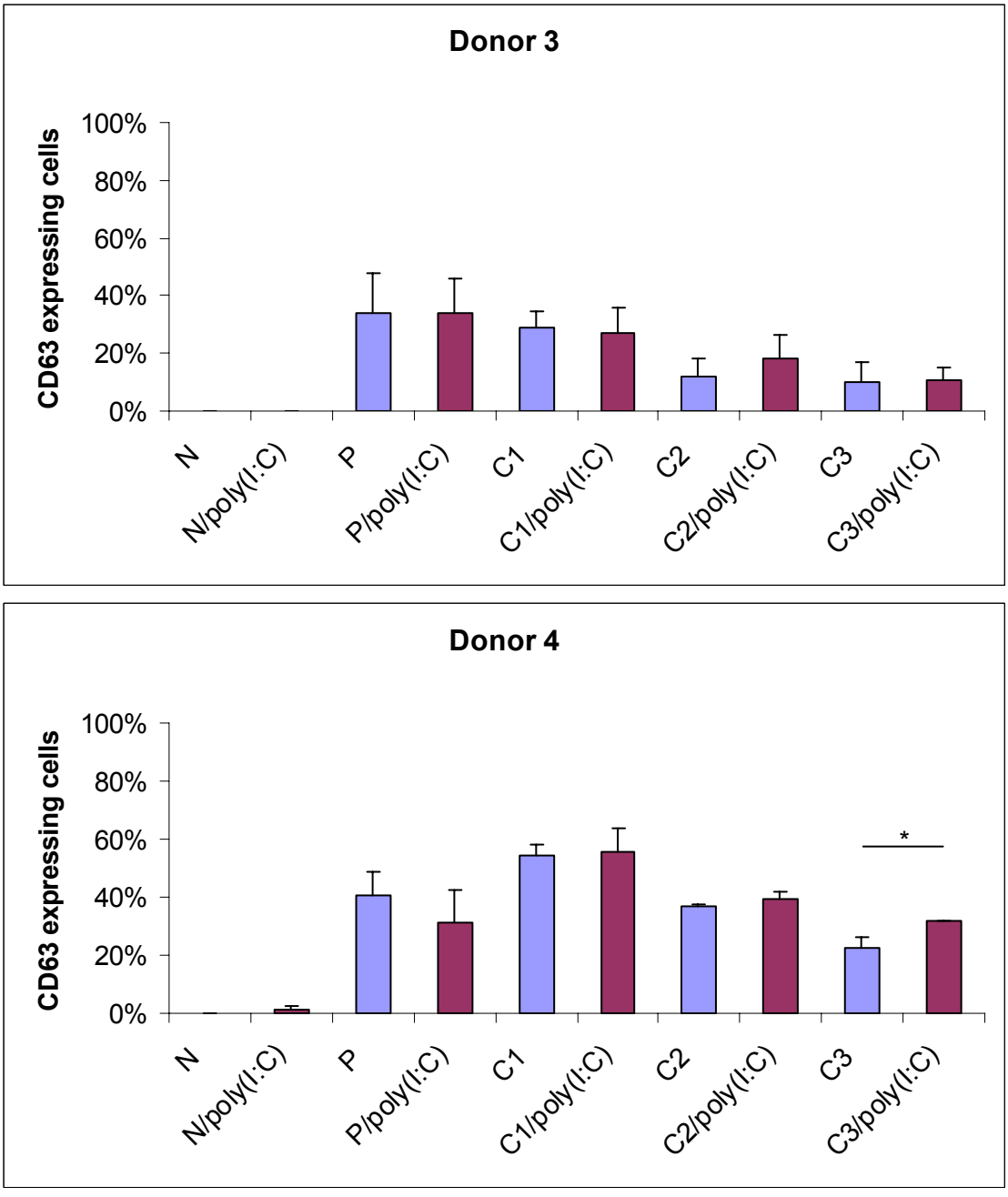
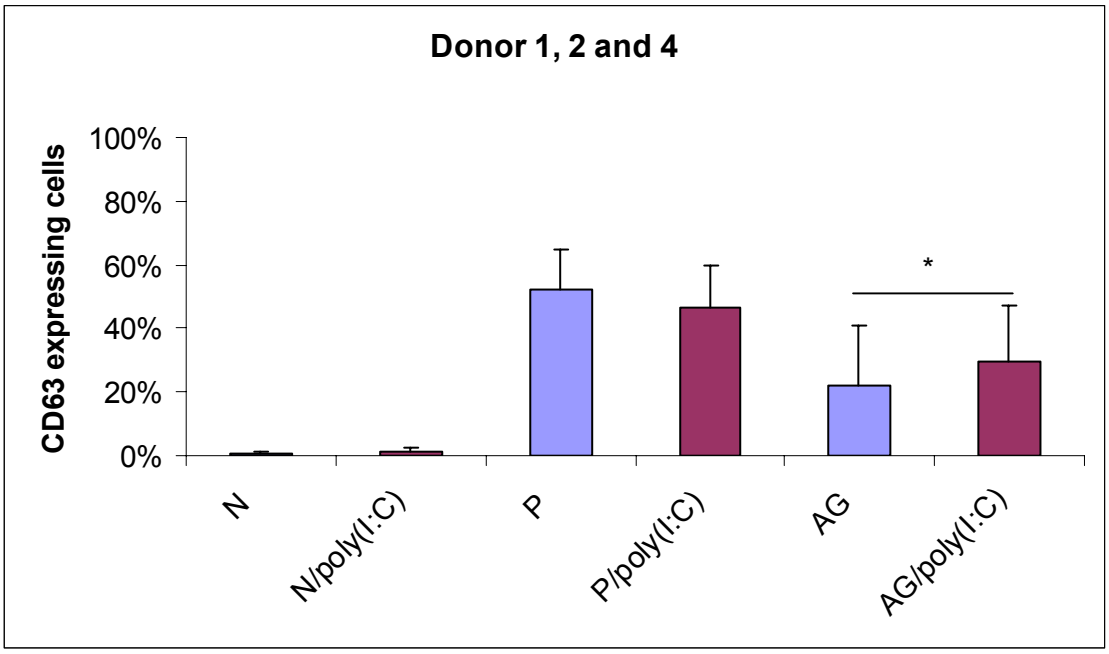


Table 10. Basophil activation after incubation with poly(I:C) and allergen. Basophil activation determined by percentage of surface CD63-expressing basophils (arithmetic mean of at least 3 independent experiments). N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Percent enhancement induced with poly(I:C) pretreatment. The level of significance is indicated by the P-value. N.s., not significant.

	without ligand (%)	**with ligand (%)**	**enhancement (%)**	**P**
Donor 1				
N	0.86	1.23	0.37	n.s.
P	56.44	50.26	-6.18	n.s.
C1	28.54	31.71	3.17	n.s.
C2	14.53	27.10	12.57	< 0.05
C3	10.45	19.99	9.54	< 0.05
Donor 2				
N	1.14	1.38	-0.02	n.s.
P	54.08	52.11	-1.97	n.s.
C1	19.13	27.89	8.76	n.s.
C2	20.53	27.66	7.13	n.s.
C3	10.86	14.96	4.1	n.s.
Donor 3				
N	0.15	0.12	-0.03	n.s.
P	34.23	34.19	-0.04	n.s.
C1	28.74	27.11	-1.63	n.s.
C2	11.95	18.04	6.09	n.s.
C3	10.23	10.63	0.4	n.s.
Donor 4				
N	0.14	1.16	1.02	n.s.
P	40.47	31.28	-9.19	n.s.
C1	54.32	55.88	1.56	n.s.
C2	37.07	39.45	2.38	n.s.
C3	22.37	31.66	9.29	< 0.05

Figure 18. Basophil activation after incubation with poly(I:C) and allergen (Donor 1, 2 and 4). Basophil activation determined by percentage of surface CD63-expressing basophils. Columns show the arithmetic mean ± standard deviation of 14 independent experiments with the 3 donors. N: negative control, P: positive control, AG: allergen (0.5 ng/ml). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.



4.1.3. Basophil activation after incubation with PGN and allergen

In all four donors treatment of basophils with heparin and IL-3 alone (negative control) or in combination with PGN did not induce CD63 surface expression. In D1 and D4 the basophil activation was lower in cells treated with PGN and anti-FcεRI mAb, than in cells treated with anti-FcεRI mAb (positive control) alone. In D2 and D3 no difference was seen between the two groups (with and without PGN) treated with anti-FcεRI mAb.

In D1 in high allergen concentration CD63 cell expression was lower in the group of PGN and allergen compared with allergen alone. Similar activation was seen between the two groups in the other concentrations. D2 showed higher expression in the group of PGN with allergen in medium and lower allergen concentration, and similar expression in the higher allergen concentration. In D3 similar CD63 expression in the two groups was shown in higher and lower allergen concentration, and higher basophil activation in cells treated with PGN and allergen in medium concentration. D4 showed significantly higher basophil activation in PGN with allergen treated cells in lower allergen concentration and similar basophil activation between the two groups in the other two allergen concentration (Figure 19, Figure 20 and Table 11).

No significant effect was shown between the two groups in basophils from D1, D2 and D4 treated with an allergen concentration of 0.5 ng/ml (Figure 21).

Taking together, with only one exception, PGN had no significant effect on CD63 expression.

Figure 19. Basophil activation after incubation with PGN and allergen (Donor 1 and 2). Basophil activation determined by percentage of surface CD63-expressing basophils. Columns show the arithmetic mean ± standard deviation of at least 3 independent experiments. N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.

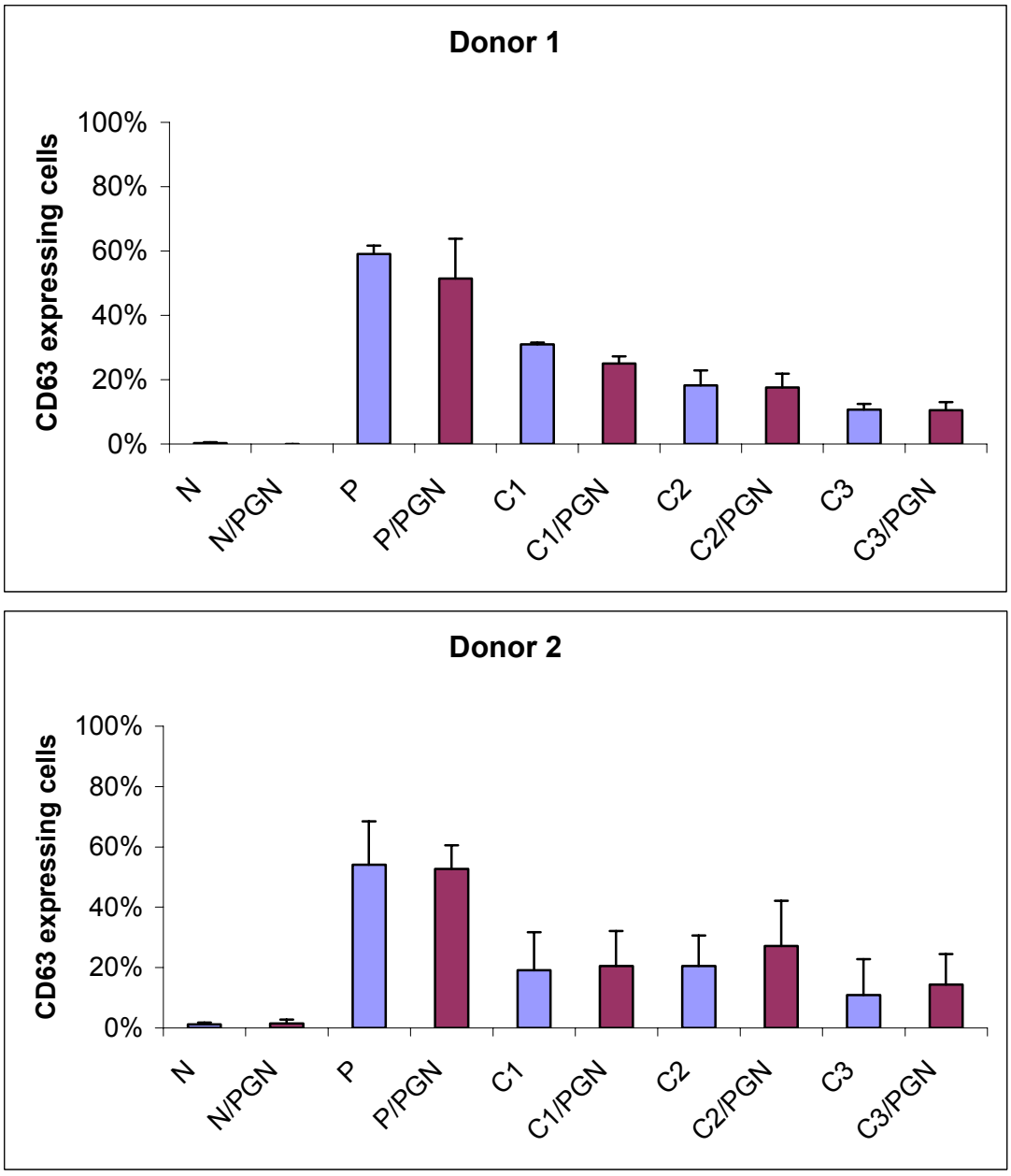
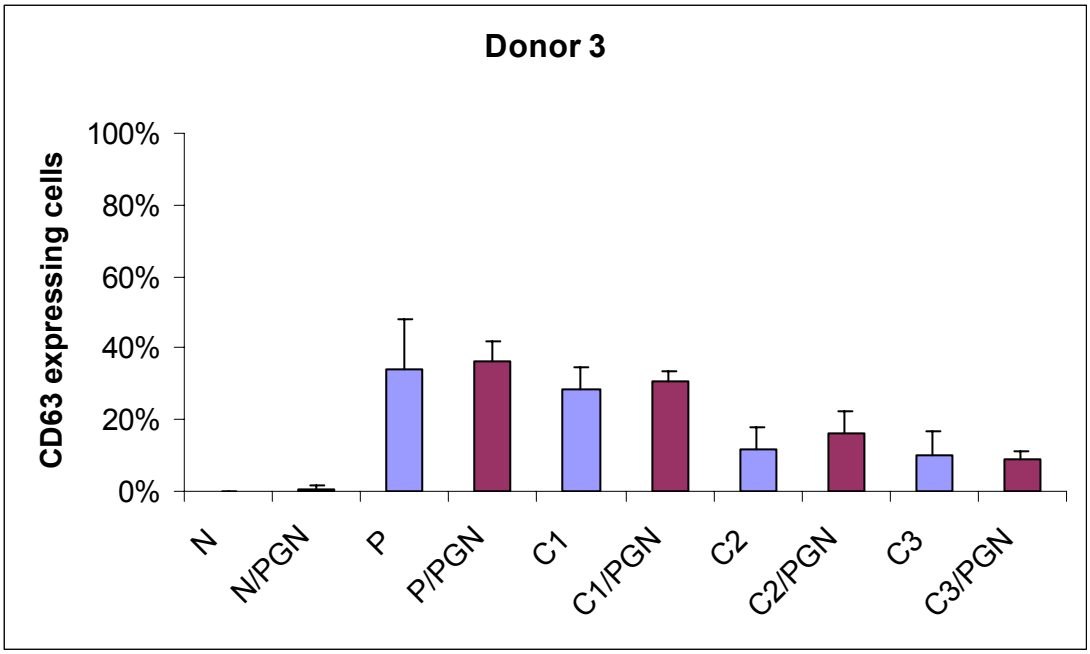


Figure 20. Basophil activation after incubation with PGN and allergen (Donor 3 and 4). Basophil activation determined by percentage of surface CD63-expressing basophils. Columns show the arithmetic mean ± standard deviation of at least 3 independent experiments. N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.



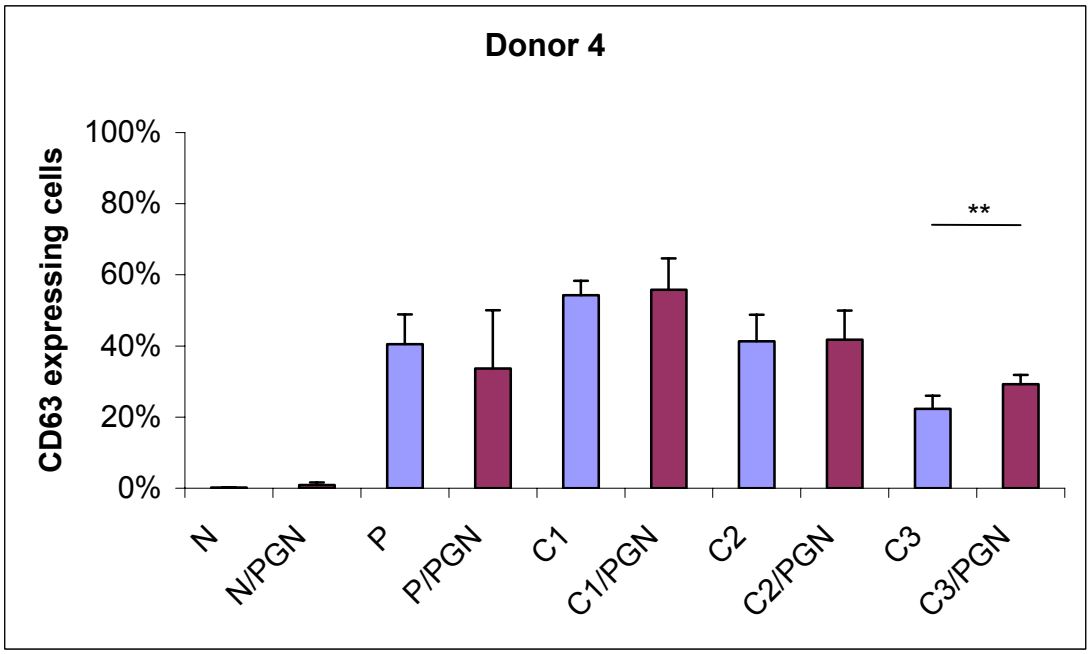
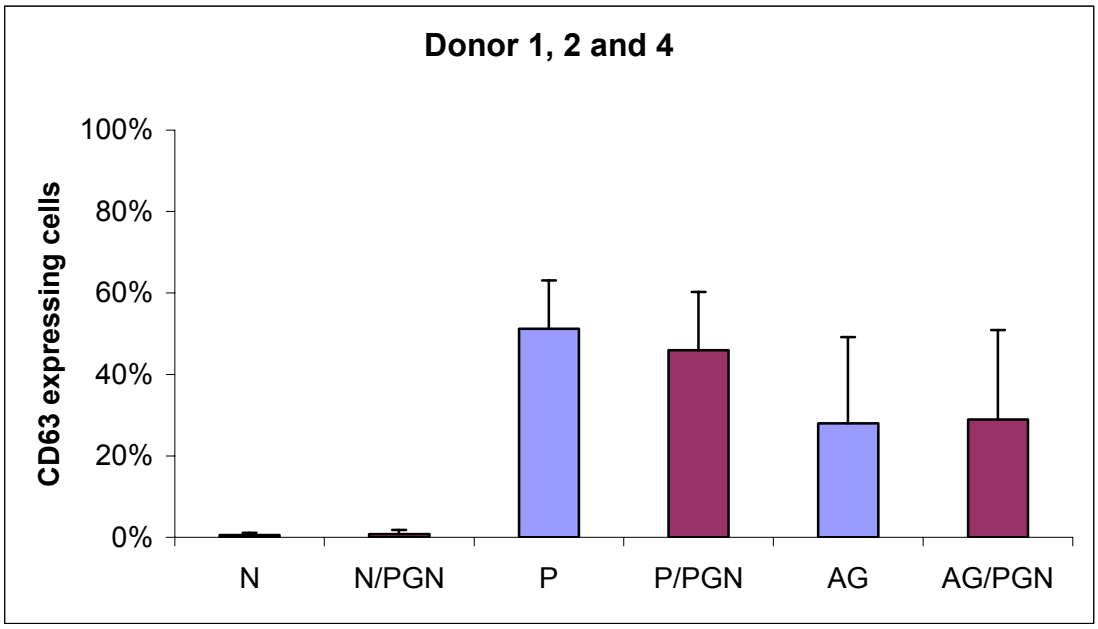


Table 11. Basophil activation after incubation with PGN and allergen. Basophil activation determined by percentage of surface CD63-expressing basophils (arithmetic mean of at least 3 independent experiments). N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Percent enhancement induced with PGN pretreatment. The level of significance is indicated by the P-value. N.s., not significant.

	without ligand (%)	**with ligand (%)**	**enhancement (%)**	**P**
Donor 1				
N	0.28	0	-0.28	n.s.
P	59.10	51.41	-7.69	n.s.
C1	30.94	24.99	-5.95	n.s.
C2	18.21	17.62	-0.59	n.s.
C3	10.69	10.48	-0.21	n.s.
Donor 2				
N	1.14	1.52	0.38	n.s.
P	54.08	52.72	-1.36	n.s.
C1	19.13	20.54	1.41	n.s.
C2	20.53	27.16	6.63	n.s.
C3	10.86	14.38	3.52	n.s.
Donor 3				
N	0.15	0.69	0.54	n.s.
P	34.23	36.50	2.27	n.s.
C1	28.74	30.46	1.72	n.s.
C2	11.95	16.16	4.21	n.s.
C3	10.23	9.02	-1.21	n.s.
Donor 4				
N	0.14	0.87	0.73	n.s.
P	40.47	33.64	-6.83	n.s.
C1	54.32	55.85	1.53	n.s.
C2	41.35	41.77	0.42	n.s.
C3	22.37	29.28	6.91	< 0.01

Figure 21. Basophil activation after incubation with PGN and allergen (Donor 1, 2 and 4). Basophil activation determined by percentage of surface CD63-expressing basophils. Columns show the arithmetic mean ± standard deviation of 9 independent experiments with the 3 donors. N: negative control, P: positive control, AG: allergen (0.5 ng/ml). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.



4.2. Sulfidoleukotriene *de novo* production

Activated basophils produce many mediators, such as histamine and leukotrienes (LT). Of the newly synthesized eicosanoid mediators, basophils primarily generate LTC_4, and its metabolites LTD_4 and LTE_4. *De novo* formation of LTC_4 can be both IgE dependent and non-IgE dependent. The latter event is usually described as pseudo-allergy.

Sulfidoleukotriene (sLT) *de novo* production was measured with use of the Cellular Antigen Stimulation Test (CAST) as described in Materials and Methods. As proposed from the manufacturer of the assay, a stimulation yield higher than 200 pg/ml sLT was regarded as positive for the reagent tested. Differences between two tested groups (with or without preincubation with TLR ligand) should have therefore been higher than 200 pg/ml, otherwise they were regarded as similar results. We also calculated the arithmetic mean of sLT production of independent experiments with 3 donors tested with the same allergen concentration. As proposed from the manufacturer of the assay, the highest measurable value was 3201 pg/ml.

4.2.1. Sulfidoleukotriene production after incubation with LPS and allergen

Treatment of basophils with heparin and IL-3 alone (negative control) or in combination with LPS did not induce sLT production. Exceptionally, in D2 a slight sLT production was observed in both treatment groups. Basically, in all 4 donors sLT production reached the highest measurable value in groups of cell treated with anti-FcεRI mAb alone (positive control) or with LPS.

Sulfidoleukotriene production of basophils treated first with LPS and then with allergen, was not higher than the production of basophils treated only with allergen. Only in D2 the sLT production was higher, but statistically not significant, in the group pretreated with the TLR ligand. In D4 the group treated only with allergen in high concentration, showed slightly higher, but not significant, sLT production (Figure 22, Figure 23 and Table 12).

No significant effect was shown between the two groups in basophils from D1, D2 and D4 treated with an allergen concentration of 0.5 ng/ml (Figure 24).

Taking together, LPS had no significant effect on sLT production.

Figure 22. Sulfidoleukotriene production after incubation with LPS and allergen (Donor 1 and 2). SLT (pg/ml) *de novo* production of basophil suspension. Columns show the arithmetic mean ± standard deviation of at least 2 independent experiments. N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.

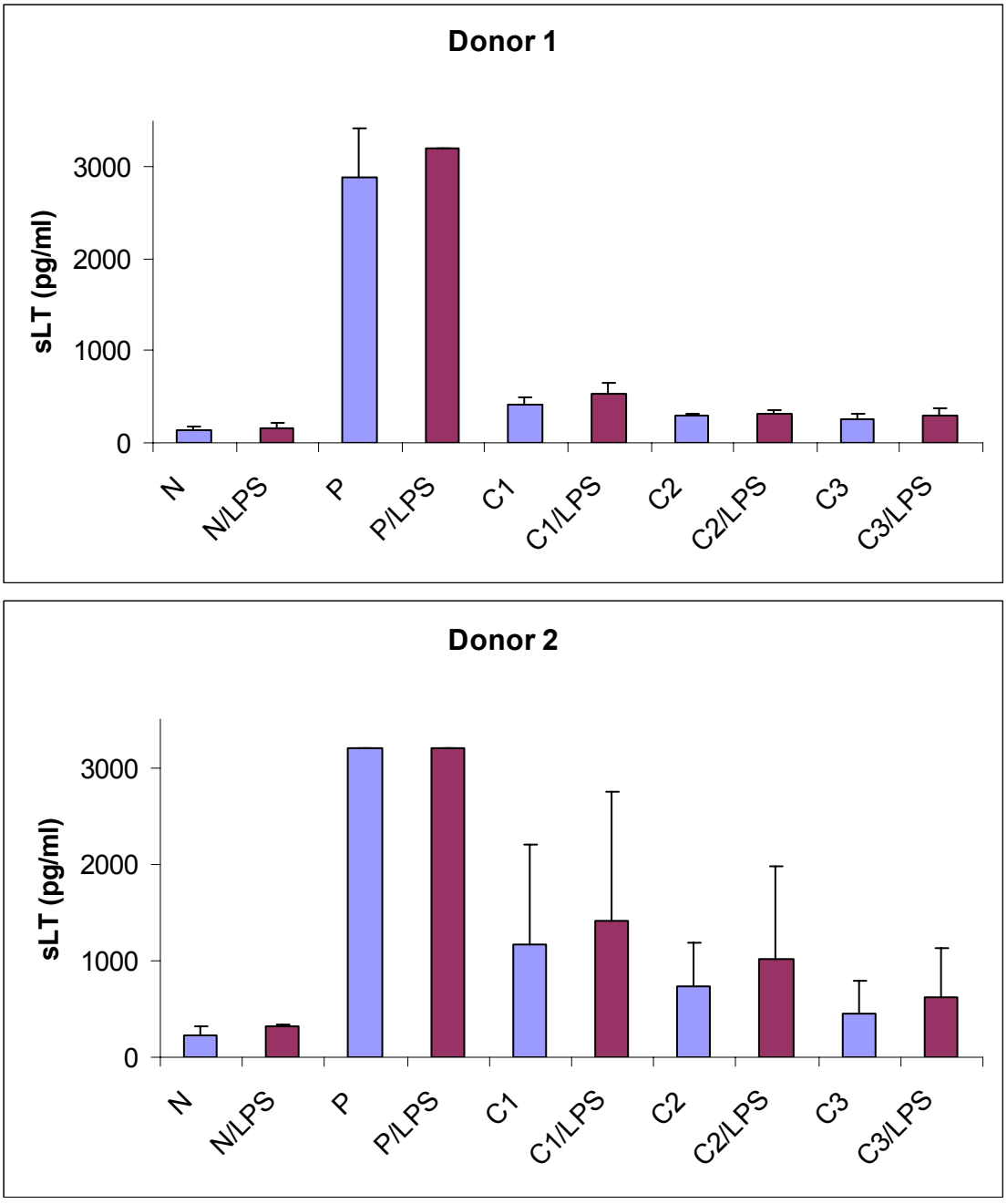
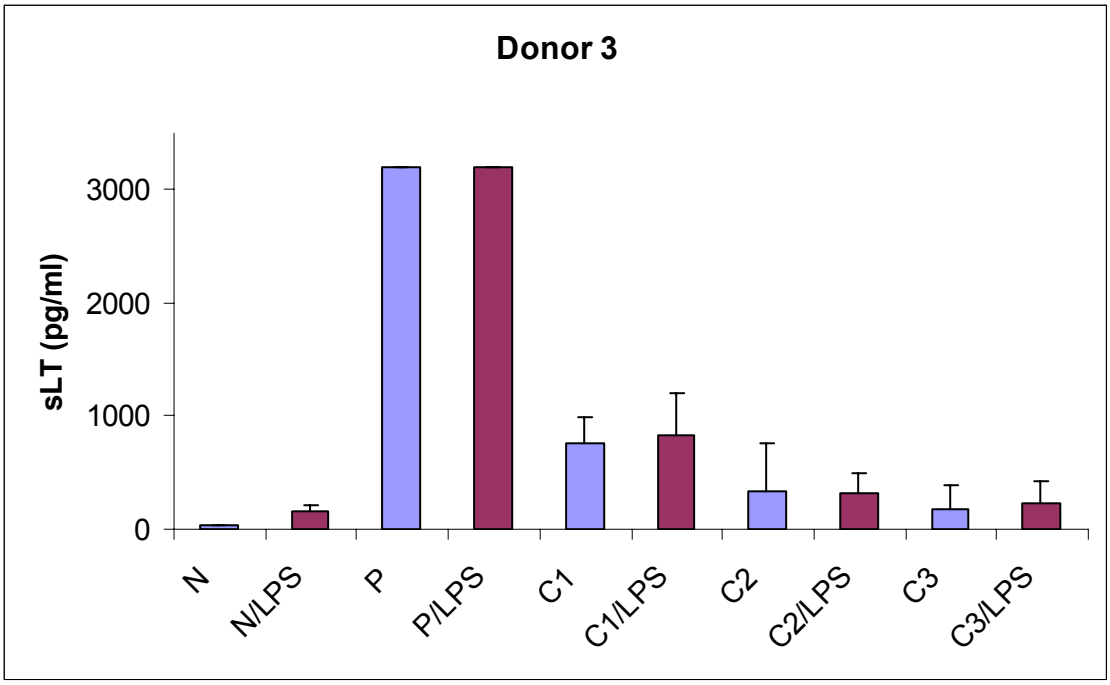


Figure 23. Sulfidoleukotriene production after incubation with LPS and allergen (Donor 3 and 4). SLT (pg/ml) *de novo* production of basophil suspension. Columns show the arithmetic mean ± standard deviation of 2 independent experiments. N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.



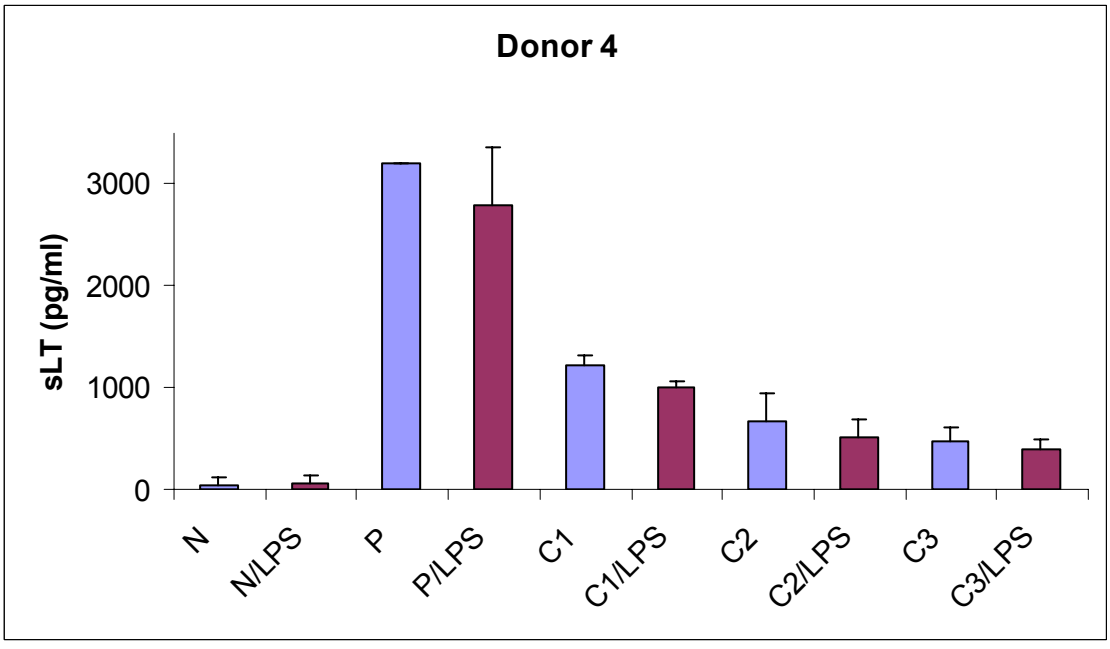
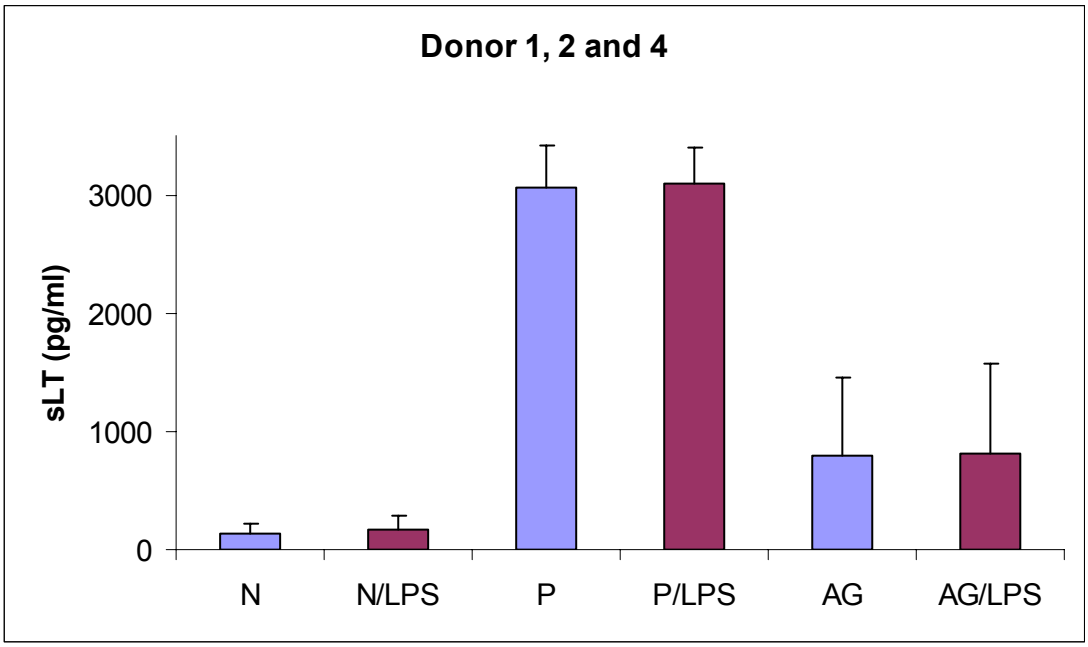


Table 12. Sulfidoleukotriene production after incubation with LPS and allergen. SLT (pg/ml) *de novo* production of basophil suspension (arithmetic mean of at least 2 independent experiments). N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Enhancement induced with LPS pretreatment. The level of significance is indicated by the P-value. N.s., not significant.

	without ligand (pg/ml)	**with ligand (pg/ml)**	**enhancement (pg/ml)**	**P**
Donor 1				
N	132	149	17	n.s.
P	2890	3201	311	n.s.
C1	424	534	110	n.s.
C2	302	322	20	n.s.
C3	251	291	40	n.s.
Donor 2				
N	231	312	81	n.s.
P	3201	3201	0	n.s.
C1	1160	1419	259	n.s.
C2	743	1013	270	n.s.
C3	457	613	156	n.s.
Donor 3				
N	32	160	128	n.s.
P	3201	3201	0	n.s.
C1	761	822	61	n.s.
C2	334	311	-23	n.s.
C3	178	237	59	n.s.
Donor 4				
N	49	56	7	n.s.
P	3201	2798	-403	n.s.
C1	1226	1010	-216	n.s.
C2	676	518	-158	n.s.
C3	480	396	-84	n.s.

Figure 24. Sulfidoleukotriene production after incubation with LPS and allergen (Donor 1, 2 and 4). SLT (pg/ml) *de novo* production of basophil suspension. Columns show the arithmetic mean ± standard deviation of 7 independent experiments with the 3 donors. N: negative control, P: positive control, AG: allergen (0.5 ng/ml). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.



4.2.2. Sulfidoleukotriene production after incubation with poly(I:C) and allergen

Treatment of basophils with heparin and IL-3 alone (negative control) or in combination with poly(I:C) did not induce sLT production. Exceptionally, a slight sLT production was observed in D2 in both groups and in D3 in group of cells preincubated with poly(I:C). Basically, in all 4 donors sLT production reached the highest measurable value in groups of cell treated with anti-FcεRI mAb alone (positive control) or with poly(I:C).

In all 4 donors similar sLT production was obtained in all allergen concentrations with or without poly(I:C). Exceptionally, in D4, cells treated with higher allergen concentration alone had higher, but not significant, sLT production compared with cells incubated with allergen and poly(I:C) (Figure 25, Figure 26 and Table 13).

No significant effect was shown between the two groups in basophils from D1, D2 and D4 treated with an allergen concentration of 0.5 ng/ml (Figure 27).

Overall, there were no significant differences in sLT production between groups treated with allergen or with allergen plus poly(I:C).

Figure 25. Sulfidoleukotriene production after incubation with poly(I:C) and allergen (Donor 1 and 2). SLT (pg/ml) *de novo* production of basophil suspension. Columns show the arithmetic mean ± standard deviation of at least 2 independent experiments. N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.

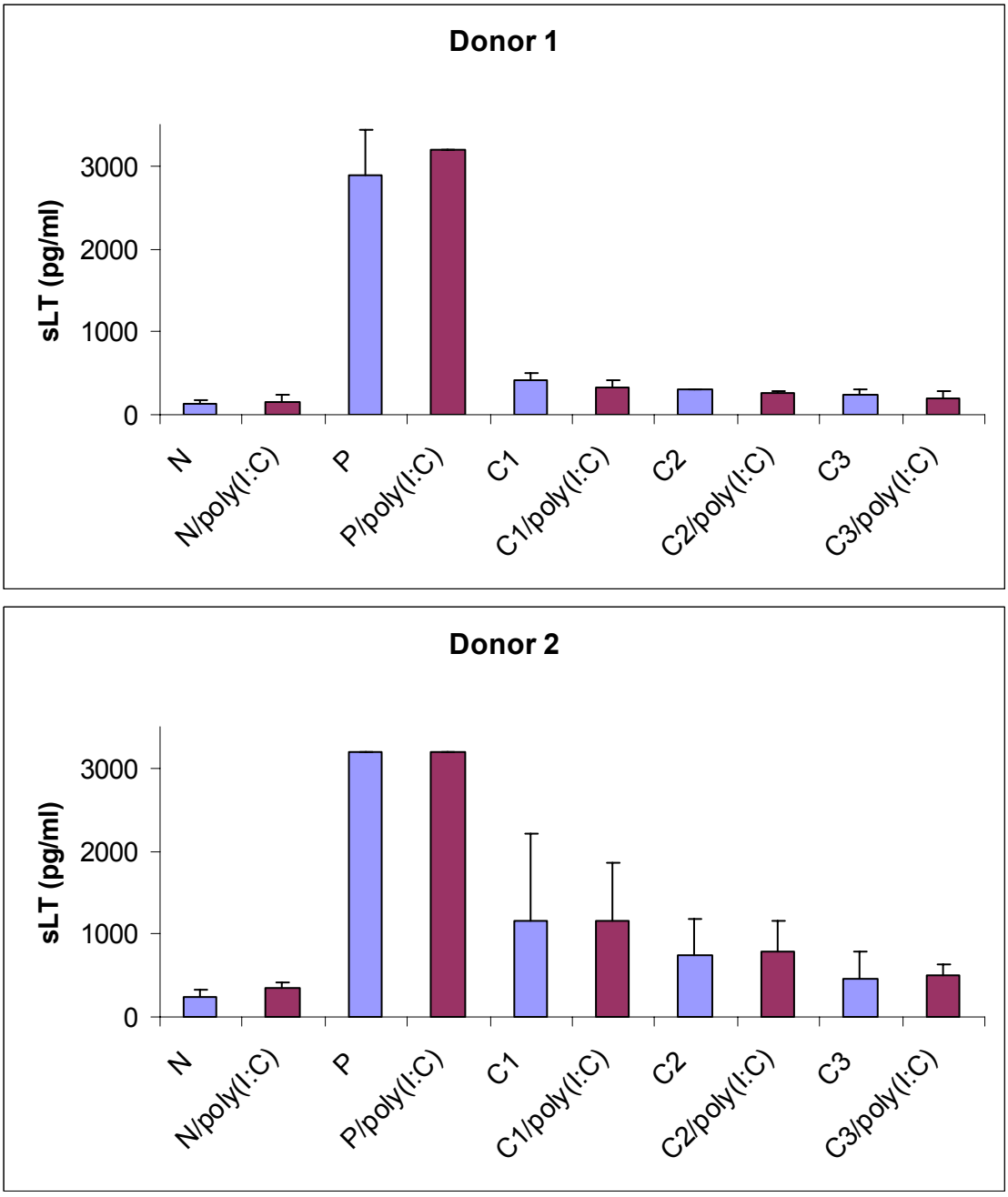
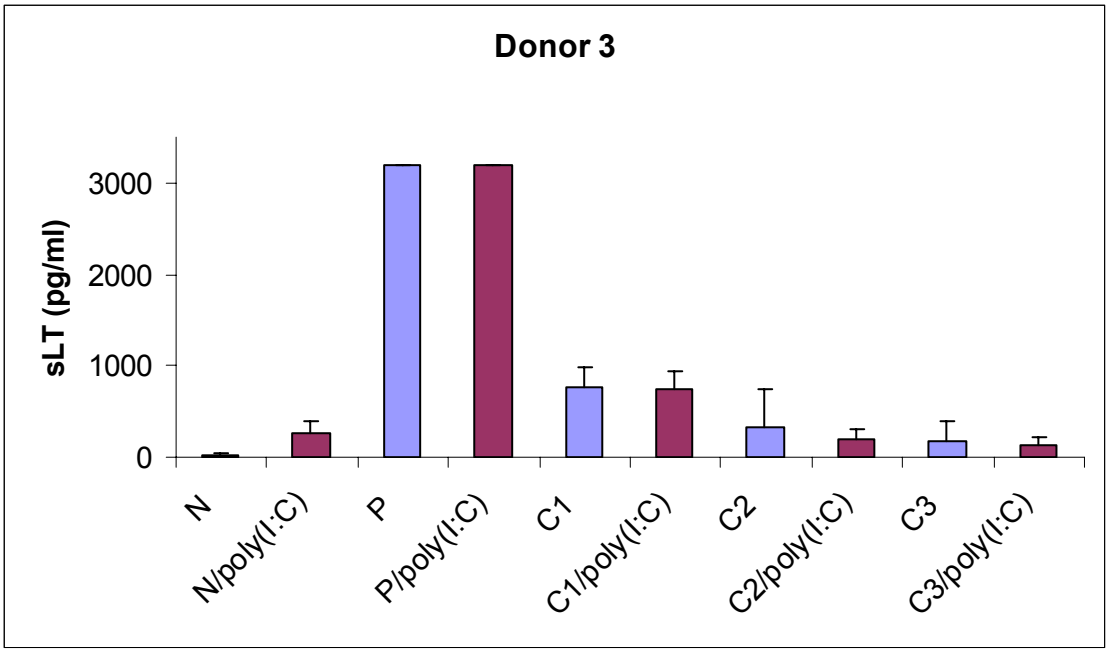


Figure 26. Sulfidoleukotriene production after incubation with poly(I:C) and allergen (Donor 3 and 4). SLT (pg/ml) *de novo* production of basophil suspension. Columns show the arithmetic mean ± standard deviation of at least 2 independent experiments. N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.



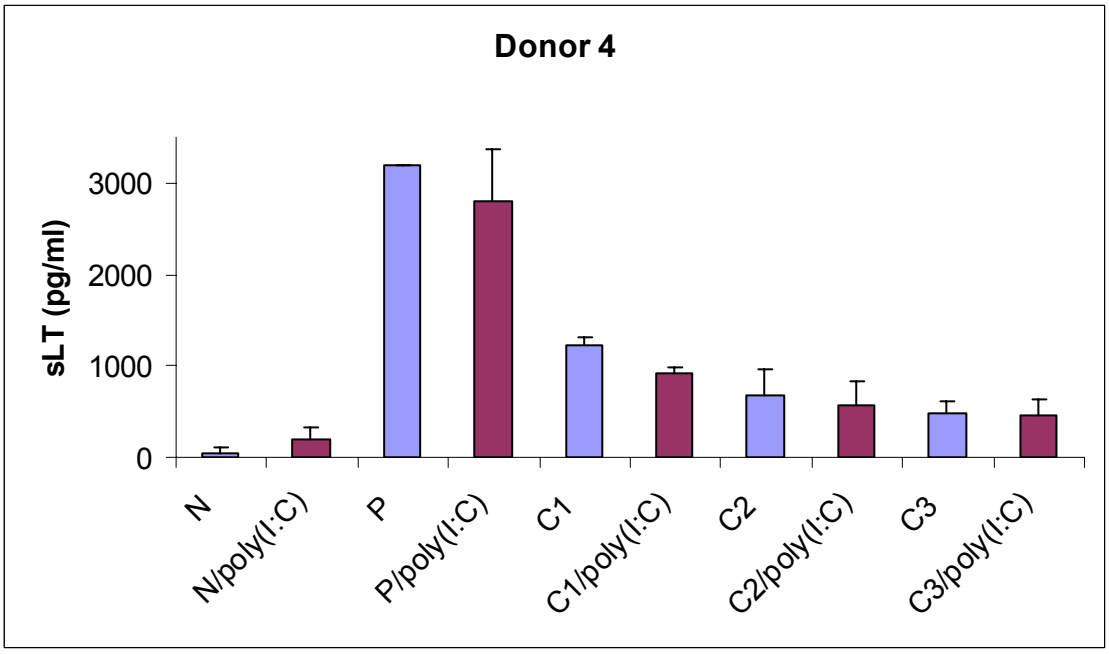
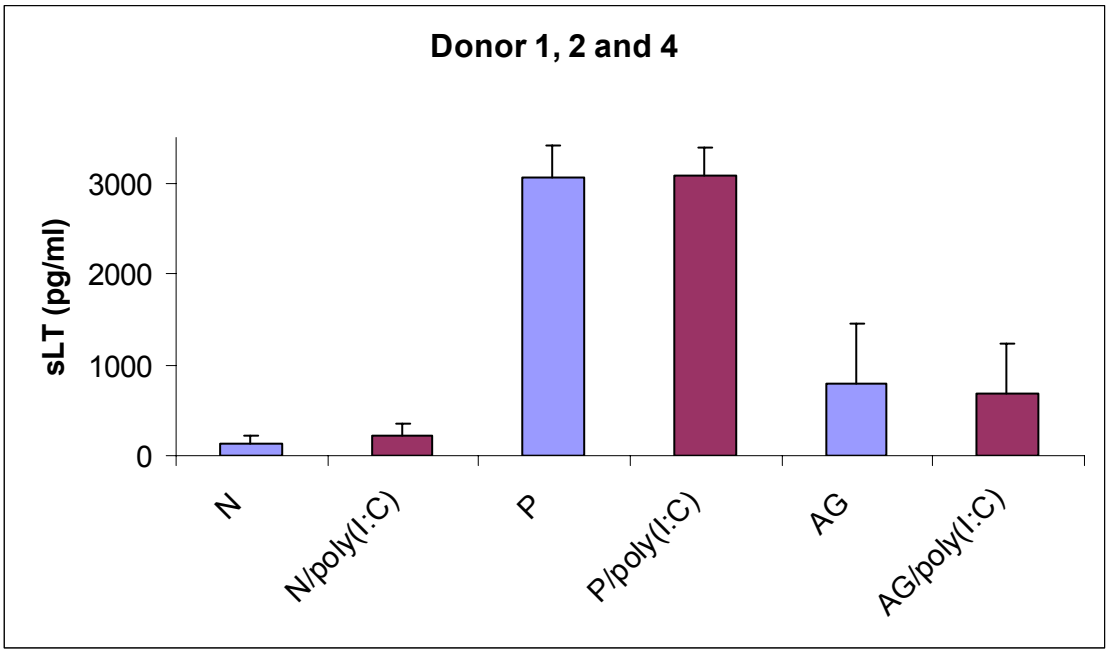


Table 13. Sulfidoleukotriene production after incubation with poly(I:C) and allergen. SLT (pg/ml) *de novo* production of basophil suspension (arithmetic mean of at least 2 independent experiments). N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Enhancement induced with poly(I:C) pretreatment. The level of significance is indicated by the P-value. N.s., not significant.

	without ligand (pg/ml)	**with ligand (pg/ml)**	**enhancement (pg/ml)**	**P**
Donor 1				
N	132	162	30	n.s.
P	2890	3201	311	n.s.
C1	424	331	-93	n.s.
C2	302	259	-43	n.s.
C3	251	203	-48	n.s.
Donor 2				
N	231	356	125	n.s.
P	3201	3201	0	n.s.
C1	1160	1169	9	n.s.
C2	743	793	50	n.s.
C3	457	498	41	n.s.
Donor 3				
N	32	259	227	n.s.
P	3201	3201	0	n.s.
C1	761	751	-10	n.s.
C2	334	203	-131	n.s.
C3	178	141	-37	n.s.
Donor 4				
N	49	204	155	n.s.
P	3201	2803	-398	n.s.
C1	1226	929	-297	n.s.
C2	676	579	-97	n.s.
C3	480	451	-29	n.s.

Figure 27. Sulfidoleukotriene production after incubation with poly(I:C) and allergen (Donor 1, 2 and 4). SLT (pg/ml) *de novo* production of basophil suspension. Columns show the arithmetic mean ± standard deviation of 7 independent experiments with the 3 donors. N: negative control, P: positive control, AG: allergen (0.5 ng/ml). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.



4.2.3. Sulfidoleukotriene production after incubation with PGN and allergen

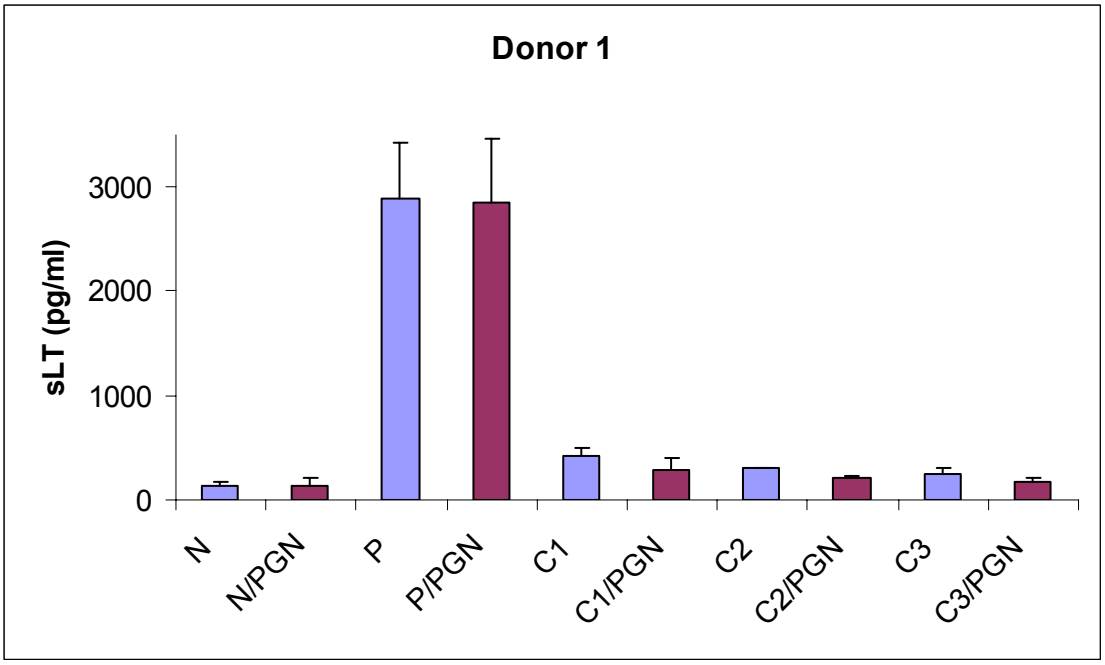
Treatment of basophils with heparin and IL-3 alone (negative control) or in combination with PGN did not induce sLT production. Exceptionally, a slight sLT production was observed in D2 in group of cells treated with heparin and IL-3 alone. Basically, in all 4 donors sLT production reached the highest measurable value in groups of cell treated with anti-FcεRI mAb alone (positive control) or with PGN.

In all 4 donors similar sLT production were obtained in all allergen concentrations with or without PGN. Exceptionally, in D2, cells treated with higher allergen concentration alone had higher, but not significant, sLT production compared with cells incubated with allergen and PGN (Figure 28, Figure 29 and Table 14).

No significant effect was shown between the two groups in basophils from D1, D2 and D4 treated with an allergen concentration of 0.5 ng/ml (Figure 30).

In conclusion, PGN had no effect on allergen- and IgE-dependent sLT release.

Figure 28. Sulfidoleukotriene production after incubation with PGN and allergen (Donor 1 and 2). SLT (pg/ml) *de novo* production of basophil suspension. Columns show the arithmetic mean ± standard deviation of at least 2 independent experiments. N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.



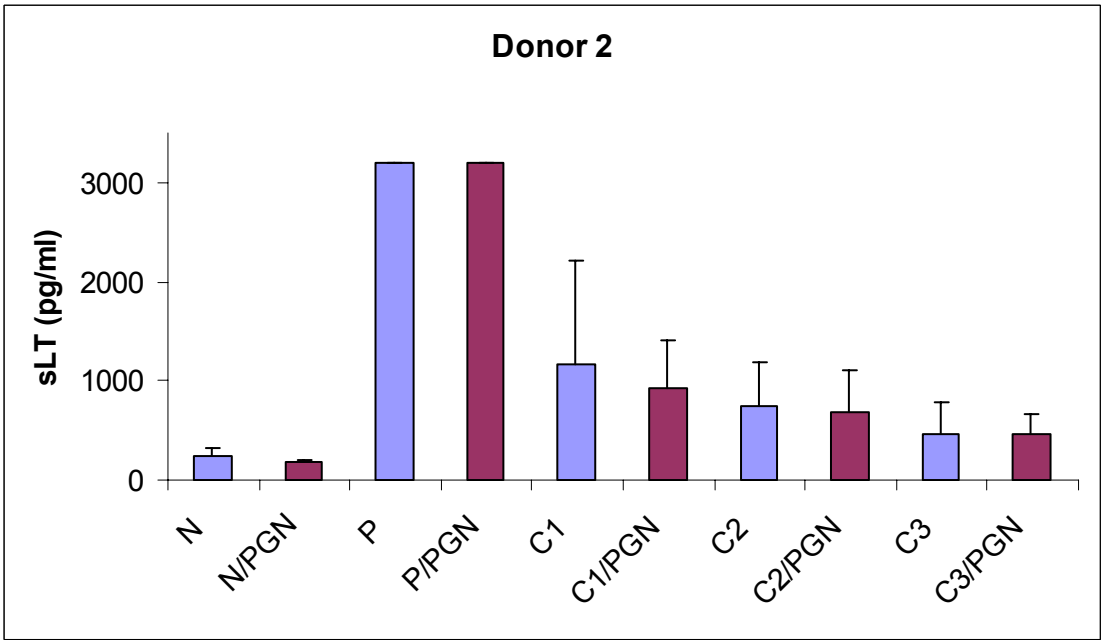


Figure 29. Sulfidoleukotriene production after incubation with PGN and allergen (Donor 3 and 4). SLT (pg/ml) *de novo* production of basophil suspension. Columns show the arithmetic mean ± standard deviation (Donor 3 (n = 2), Donor 4 (n = 1)). N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Level of significance: asterisk, P < 0.05; two asterisks, P < 0.01; three asterisks, P < 0.001. If nothing indicated: no significant difference.

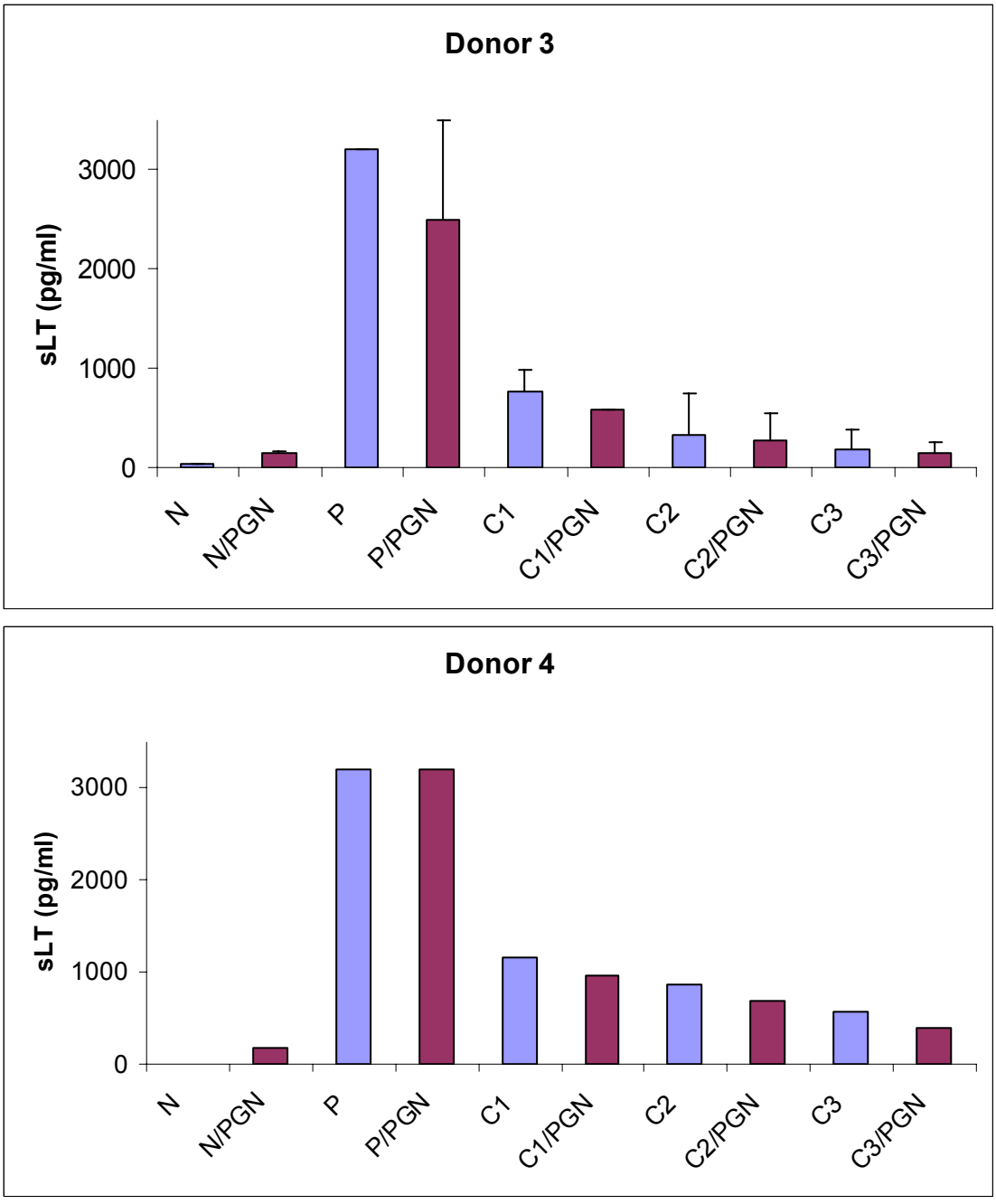
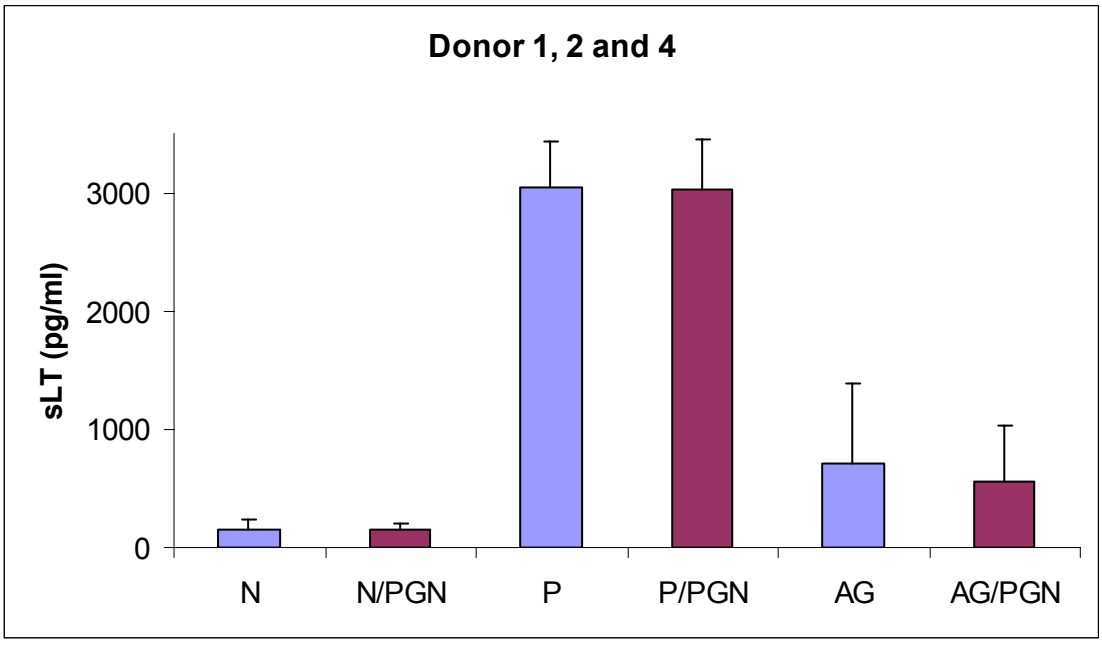


Table 14. Sulfidoleukotriene production after incubation with PGN and allergen. SLT (pg/ml) *de novo* production of basophil suspension (arithmetic mean. Donor 1 (n = 3), Donor 2 (n = 2), Donor 3 (n = 2), Donor 4 (n = 1)). N: negative control, P: positive control, C1/C2/C3: higher/medium/lower allergen concentration respectively (see Table 8). Enhancement induced with PGN pretreatment. The level of significance is indicated by the P-value. N.s., not significant.

	without ligand (pg/ml)	**with ligand (pg/ml)**	**enhancement (pg/ml)**	**P**
Donor 1				
N	132	136	4	n.s.
P	2890	2856	-34	n.s.
C1	424	286	-138	n.s.
C2	302	215	-87	n.s.
C3	251	169	-82	n.s.
Donor 2				
N	231	178	-53	n.s.
P	3201	3201	0	n.s.
C1	1160	929	-231	n.s.
C2	743	684	-59	n.s.
C3	457	472	15	n.s.
Donor 3				
N	32	152	120	n.s.
P	3201	2492	-709	n.s.
C1	761	579	-182	n.s.
C2	334	278	-56	n.s.
C3	178	142	-36	n.s.
Donor 4				
N	6	179	173	n.s.
P	3201	3201	0	n.s.
C1	1158	972	-186	n.s.
C2	872	694	-178	n.s.
C3	576	393	-183	n.s.

Figure 30. Sulfidoleukotriene production after incubation with PGN and allergen (Donor 1, 2 and 4). SLT (pg/ml) *de novo* production of basophil suspension. Columns show the arithmetic mean ± standard deviation of 6 independent experiments with the 3 donors. N: negative control, P: positive control, AG: allergen (0.5 ng/ml). Level of significance: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. If nothing indicated: no significant difference.



5. Discussion

The identification of Toll-like receptor (TLR) molecules has sparked renewed interest in innate immunity through the notion that these proteins play a critical role in directing the course of acquired immnunity, including that associated with allergic disease. Eleven of these receptors (TLR1-11) have been identified, and there is increasing evidence that they are selectively expressed among immune cells, where they induce a variety of responses (55, 117).

Allergic reactions are dominated by the preferential development of specific TH_2 responses against innocuous antigens in atopic individuals. This can reflect alterations in innate immune mechanisms. Toll-like receptors have evolved as key molecules in innate and adaptive immunity. Their activation by structurally distinct exogenous or endogenous ligands present at the cell microenvironment plays a critical role in antimicrobial defense. The global view is that TLR activation induces antigen-presenting cells to produce cytokines that favor TH_1 – type immune responses, suggesting that it might prevent the development of deleterious TH_2 responses in allergy. Until recently, most TLR/ligand interactions had been shown to favor TH_1-like responses rather than promoting TH_2 responses commonly associated with allergic disease.

However, this concept was recently challenged in rodent models, in which both TLR ligand concentration and route of immunization were shown to play important roles in determing whether TH_1 or TH_2 immune responses develop (27, 36, 91). On the basis of epidemiological studies and recent data, it has been established that TLRs play a role in the development of TH_2 responses. A pathogenetic role for TLRs and TLR ligands cannot be ruled out, especially once allergic inflammation is established.

Depending on the frame of the interaction with pathogens, the role of TLRs could be dual: in addition to the potentially important role of these receptors in the induction phase of an allergic phenotype, a TLR-driven immune response might also have an essential role in disease exacerbation (42).

The basophil has traditionally been viewed primarily as an effector cell in allergic diseases, such as seasonal allergic rhinitis. More recently, it has been found that human basophils express high levels of TLR2 and TLR4 compared with other granulocytes, raising the possibility of TLR involvement in mediating basophil responses (10, 94). Peptidoglycan (PGN) and lipopolysaccharide (LPS) are respective ligands for TLR2 and TLR4. There is no data available concerning the expression of TLR3 on human basophils, which recognizes viral dsRNA as modeled by the synthetic dsRNA analogue polyinosine-polycytidylic acid [poly(I:C)]. A recent study (65) showed activation of human mast cells by dsRNA, providing evidence for activation through TLR3. The same study (65) showed that human mast cells express TLR3. Another study (6) investigated the expression of TLRs in established murine lymphoid and myeloid cell lines. They found that TLR3 is not expressed by cell lines of the mast cell and basophil lineage.

It has long been recognized that viral or bacterial infection of the upper respiratory track can exacerbate the symptoms of allergic rhinitis or asthma (29, 56) which may suggest direct or indirect implications of cells such as basophils and mast cells.

In this study, we investigated the effect of LPS, poly(I:C) and PGN on basophils from subjects with allergy. More precisely, we tested whether these TLR ligands enhance CD63 expression of basophils in response to pollen allergen (Timothy grass). Furthermore, we tested whether LPS, poly(I:C) and PGN augment sLT production from basophils stimulated with allergen.

Among the several cytokines and growth factors that are capable of potentiating or priming basophils (IL-3, IL-5, GM-CSF, and NGF), IL-3 is widely used in *in vitro* basophil activation assays (21, 95). We showed that basophils treated with IL-3 did not induce CD63 expression. In keeping with a previous study (24), in which LTC_4 in basophils cultured with IL-3 alone was generally not detectabe or minimal, we showed that IL-3 alone had no effect on sLT production from basophils.

Toll-like receptor 4 was the first human homologue of Toll to be described (77) and was subsequently characterized as a receptor for LPS signaling (87). We demostrated that LPS alone had no effect on CD63 expression and sLT production on basophils treated with IL-3.

We investigated the effect of LPS on basophils from donors with grass pollen allergy. We demostrated that preincubation of basophils with LPS and subsequent stimulation with Timothy grass pollen extract significantly enhanced the CD63 expression compared with allergen alone (see Figures 13-15 and Table 9). With one exception (otherwise not statistically significant), pretreatment with LPS did not affect allergen–mediated basophil secretion of sLT.

Our findings confirmed the results from Bieneman et al (10), who showed that pretreatment of basophils with LPS alone did not affect anti-IgE-mediated basophil secretion, especially histamine, LTC_4, and IL-4.

Bieneman et al (10) also showed that basophils did not react to LPS in assessing nuclear localization of NFκB as a measure of functional TLR receptor responses and concluded that basophils were unresponsive to LPS despite expressing TLR4. As possible explanation they propose that these cells do not normally express the coreceptor CD14 (107). A previous study from Sabroe et al (94) also showed that

basophils did not respond to LPS. That study was performed measuring other parameter such as CD11b expression.

LPS, although *per se* having no effect on basophil CD63 expression or sLT production, can augment allergen-dependent CD63 basophil expression (see Figures 13-15 and Table 9). This effect of LPS on IgE-mediated CD63 expression has never been described. The mechanism of this increase of allergen-dependent CD63 expression is not yet known. LPS may act by modulating basophil cytokine secretion and mediator release independently of NFκB activation.

Accordingly, our results suggest that LPS induces signals downstream from those directly linked to sLT release but is still capable of basophil activation.

We can not explain this phenomenon easily. A possible answer can be the low ligand concentration. Maybe higher concentrations of ligand are necessary in order to augment not only basophil activation, but also production of leukotrienes. However, two recent studies (34, 96) showed that correlation between the basophil activation test (using flow cytometry) and the leukotriene release (using CAST) is suboptimal.

Toll-like receptor 3 constitutes a separate subfamily within the mammalian TLRs that is characterized by intracellular expression (41). Ligation of TLR3 senses dsRNA (111). TLR3 activation was achieved by the TLR3 ligand poly(I:C) (5), a synthetic analogue of viral dsRNA.

We demostrated that poly(I:C) had no effect on CD63 expression and sLT production on basophils treated with IL-3 alone. We investigated the effect of poly(I:C) on basophils from allergic donors. We provided evidence that, preincubation of basophils with poly(I:C) and subsequent stimulation with allergen significantly enhanced CD63 expression compared with allergen alone (see Figures 16-18, Table

10). As seen with LPS, poly(I:C) had not additional effect on sLT production from basophils stimulated with allergen.

Viruses, dsRNA or poly(I:C) are recognized in a TLR3-dependent and a TLR3-independent manner (5, 116). Whether human basophils express TLR3 or not is still unknown. A study (6) with murine lymphoid and myeloid cell lines showed that TLR3 is not expressed by cell lines of the mast cell and basophil lineage. Thus, we can not exclude that other receptors than TLR3 could also be involved. However, other study (65) showed activation of human mast cells by dsRNA, providing evidence for activation through TLR3. The same study (65) showed that human mast cells express TLR3.

Our data suggest that either in a TLR3-dependent or in TLR3-independent way, basophils respond functionally to poly(I:C). As previously discussed with LPS, the reason why enhanced basophil CD63 expression, thus basophil activation, failed to show enhanced sLT production, is unclear. As mentioned before the low ligand concentration may be a reason. Furthermore, as shown in previous studies (34, 96) the correlation between basophil activation test (using flow cytometry) and leukotriene release (using CAST) is suboptimal.

TLR2 has previously been shown to mediate PGN recognition. However, NOD1 and NOD2 have recently been shown to recognize motifs found in the layer of PGN. It is possible that TLR2 recognizes lipoprotein contamination in the PGN layer (22, 26, 48, 49, 54, 105, 108).

In our study, we showed that PGN did not affect CD63 expression or sLT production on basophils treated with IL-3. Our findings confirmed previous data from Bieneman et al (10), who showed that although basophils reacted to PGN in assessing nuclear localization of NFκB and likewise, in direct response to PGN secreted IL-4 and IL-13,

PGN did not induce histamine or LTC_4 production from basophils. Furthermore, we provided evidence that PGN did not augment basophil CD63 expression in response to allergen-dependent activation.

Bieneman et al (10) found that treatment with PGN and anti-IgE antibody enhanced the secretion of histamine, LTC_4 and IL-4 compared with activation with anti-IgE alone. We found that PGN did not affect the sLT production, when these cells were pretreated with this ligand and then stimulated with anti-FcεRI mAb or allergen. Bieneman et al (10) otherwise used anti-IgE antibody in their study and not anti-FcεRI mAb or allergen as we did. Furthermore, the longer incubation time with anti-IgE used in their experiments, could be another possible reason for the discrepancies.

Taking together, this study clearly demonstrates that none of the three tested TLRs ligands alone (without allergen) can either provoke CD63 basophil expression or sLT production from basophils. Furthermore, LPS and poly(I:C) augment CD63 basophil expression in an allergen-dependent way, however not sLT release. PGN has no effect on CD63 expression or sLT production induced by allergen.

Over the past century, many immunologists have studied endotoxin/LPS and found their experiences to be both scientifically fertile and frustrating. Although studies of endotoxin have enlightened our understanding of the immune response to microbes, each door of knowledge has opened to reveal paradoxes that have challenged our paradigms. In keeping with this legacy, there is mounting evidence that environmental exposure to endotoxin has an ambiguous relationship with allergy. Somehow, endotoxin exposure aggravates allergy and might have allergy-protective effects.

Yet allergies directly resulting from microbial infections are rare unless an underlying condition already exists. In this regard, infections are commonly associated with exacerbation of allergy and of allergic disease (68).

The data of this study provided evidence that innate immune responses mediated through LPS and poly(I:C) may play a role in augmenting allergic reactions. Priming is the capacity for molecules (IL-3, IL-1, IL-5, GM-CSF, stem-cell factor, nerve growth factor, histamine releasing factor, and IFN-γ) that cannot maximally activate basophils on their own to augment FcεRI-mediated activation. We conclude that at least LPS and poly(I:C) have a similar priming activity, but the mechanism of action is not yet precisely known. The ability of PGN to target basophils for IL-4 secretion (10) favours farther the theory proposed that basophils could play an important role in promoting and amplifying the TH_2-dependent responses exemplified in allergic disease (99).

Our findings support epidemiological studies and recent data, that TLRs play a role in the development of TH_2 responses. However, more information is needed to fully understand the mechanism of TLR involvement and the implication of immune cells, especially basophils that express TLRs in the TH_1/TH_2 cytokine profile.

Recently, growing amounts of data suggest the ability of certain individuals to respond properly to TLR ligands may be impaired by single nucleotide polymorphisms within TLR genes, resulting in an altered susceptibility to, or course of, infectious or inflammatory disease (98). This finding could help to explain the individual differences found in the present study between the 4 donors with each of the TLR agonists tested.

A clearer understanding of all these mechanisms will allow us to better appreciate how the host adaptive immune system communicates with the environment and will

provide potential ways by which TLRs and their signaling pathways can be used as targets for therapeutic intervention.

Further studies will have to elucidate the level of expression of the various TLRs on human basophils, the particular role and signalling response mediated by these TLRs, and their relative importance in health and disease.

More research is also needed to address the fundamental question concerning the true physiologic role of basophils in the immune system.

6. Summary

Basophils express a complete and functional FcεRI receptor ($\alpha\beta\gamma_2$), cross-linking of which leads to basophil activation. Activated basophils express on their surface CD63, a tetraspan antigen and produce many mediators, such as histamine and leukotrienes.

The recently-discovered class of Toll-like receptors (TLRs) plays an essential role in the complex defence system against microorganisms. Toll-like receptors are the first to detect potential pathogens, initiate immune responses and form the crucial link between the innate and acquired immune systems. Toll-like receptors play an important role in the pathophysiology of infectious diseases, inflammatory diseases and possibly play role in autoimmune diseases and allergic diseases.

Although allergies directly resulting from microbial infections are rare, infections are commonly associated with exacerbations of allergic diseases. Based on this common clinical observation, we investigated the effect of lipopolysaccharide (LPS), polyinosine-polycytidylic acid [poly(I:C)] and peptidoglycan (PGN) on basophils from subjects with allergy. More precisely, whether these TLR agonists enhanced CD63 expression of basophils in response to pollen allergen (Timothy grass). Furthermore, we tested whether LPS, poly(I:C) and PGN augment sulfidoleukotriene (sLT) production from basophils stimulated with allergen.

The data of our study suggested that LPS and poly(I:C), but not PGN are able to enhance basophil activation in an allergen-dependent way. It is still not known if these enhancements are Toll-like receptor-dependent or not.

Our study also clearly showed that TLR ligands, such as LPS, poly(I:C) and PGN alone have no effect on basophil CD63 expression. Furthermore we showed that

LPS, poly(I:C) and PGN do not affect allergen mediated basophil secretion of sLT and likewise we demonstrated that these TLR ligands alone have no effect on secretion of sLT from basophils.

The data of this study provided evidence that innate immune responses mediated through LPS and poly(I:C) may play a role in augmenting allergic reactions. Priming is the capacity for molecules that cannot maximally activate basophils on their own to augment FcεRI-mediated activation. We conclude that at least LPS and poly(I:C) have a similar priming activity, but the mechanism of action is not yet known.

Our findings support epidemiological studies and recent data, that TLRs play a role in the development of TH_2 responses. However, more information is needed to fully understand the mechanism of TLR involvement and the implication of immune cells, especially basophils, that express TLRs in the TH_1/TH_2 cytokine profile.

Further studies will have to elucidate the expression of various TLRs on human basophils, the particular role and signalling response mediated by these TLRs, and their relative importance in health and disease.

More research is also needed to address the fundamental question concerning the true physiologic role of basophils in the immune system.

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9. Curriculum vitae

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1990-1997	Bachelor in Medicine, Good
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2000-2001	General practioner in greek army
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Congresses – Seminars

Participations and presentations in many international and national congresses

Languages

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German	fluent
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Activities – Interests

Literature, Music, Visual Arts, Cinema

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