Immunoglobulin free light chains in inflammatory diseases

New findings on FLCs fitted into current concepts of immune regulation

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ISBN: 978-90-393-5721-7

Cover design: Evert Buis

Printed by: GVO | Ponson & Looijen B.V., Ede, The Netherlands

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Immunoglobuline vrije lichte ketens in ontstekingsziekten

Nieuwe inzichten in de biologie van FLCs in relatie tot immuunregulatie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
woensdag 8 februari 2012 des middags te 2.30 uur

door

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geboren op 5 maart 1984 te Winterswijk

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The publication of this thesis was financially supported by:

Danone Research – Centre for Specialised Nutrition

Clinquest

J.E. Jurriaanse Stichting

Contents

1

General introduction

Part of this chapter is published in *Current Pharmaceutical Design*, 2011

Immunobiology of antigen-specific immunoglobulin free light chain in chronic inflammatory diseases

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Many of the pathological mechanisms that mediate inflammatory related disorders ranging from allergy to cancer are still poorly understood currently and subject to extensive research nowadays. Interestingly, the mast cells are increasingly recognized as critical players in the sensitization and effector phase of chronic inflammatory immune responses [1]. Mast cells are highly versatile cells, which are able to secrete a plethora of mediators upon activation and thereby exerting immunomodulatory functions [2-13]. Depending on the microenvironment and the activating signals, mast cells can direct the immune response into either pro- or anti-inflammatory directions. Binding of IgE to their high affinity receptor (FcεRI) and subsequent cross-linking of receptor bound IgE is the best and most powerful known mechanism of mast cell activation [14, 15]. However, also a different route of antigen-specific activation has been described, namely via immunoglobulin free light chains (FLCs). About three decades ago the research was initiated when an unknown spleen supernatant factor showed to be able to induce an antigen-specific mast cell activation independent of IgE [16, 17]. Initially, this factor was thought to come from T cells, and therefore referred to as "T cell factor" (TCF) and later as the product of contact sensitization with picryl chloride (PCl) as PCl-Factor (PCl-F). In 2002, it was described that the active proteins within these TCFs were, at least partly, immunoglobulin free light chains, and consequently a product of B cells/plasma cells instead of T cells [18, 19]. In this chapter, the research concerning FLCs will be described from the start to subsequent findings that lead to the initiation of this thesis. Furthermore, because of the seemingly important role of mast cells in FLC-mediated effects, the role of mast cells in mediating different pathologies and preclinical models involved in mast cell research will be addressed in more detail. Following on the concepts of FLC research presented in this chapter, a brief overview of this thesis is outlined in the final paragraph.

Biological functions of supernatant factors later ascribed to FLC

A role for mast cells in contact sensitivity (CS) and delayed type hypersensitivity (DTH), in fact a required role, had never before been contemplated when work began on the mast cell activator T cell factor (TCF). The first studies suggesting this were pharmacological, and indicated for the first time that serotonin (5-HT) was required for the elicitation of CS and DTH [20]. In mice, 5-HT is a powerful vasoactive mediator, and compared with histamine that also is produced by mast cells and also stored in their granules, is 1000 times more powerful in mediating vasoactivity [21]. Comparable vascular activation attempted with histamine results in severe acidosis and death. Fluorescent cytochemistry and uptake of ^{3}H-5-HT were used to determine the possible sources of 5-HT in CS and DTH. These techniques indicated that mast cells were a possible source, as well as platelets [20, 22]. In fact in mice, mast cells and platelets are the only source of 5-HT outside of the nervous system. Further, attempts to

elicit DTH in immunized mice by challenge injection of antigen in the flank skin, the preferred site in guinea pigs and rats, failed. This correlated with a paucity of mast cells at that site. In contrast, elicitation of CS in the ears and DTH in the foot pads gave far superior results, and it was shown that these sites had particularly high numbers of 5-HT containing mast cells [20]. Further, mast cell-deficient mice have defective elicitation of CS and DTH and impaired recruitment of effector T cells locally to elicit these responses [23]. Finally, electron microscopic analysis indicated that mast cells in CS/DTH were undergoing partial piecemeal degranulation instead of the standard IgE-mediated anaphylactic exocytosis [22-24], suggesting differential release of mediators. Functional studies showed preferential release of 5-HT compared to histamine; two mediators seemingly stored in the same granule, suggesting that indeed there was differential release [25-28] and thus the mechanism of release of mediators was different and special in CS/DTH.

At this point it was still unclear how the mast cells were activated in CS/DTH. A mixture of four day PCl immune spleen and lymph node cells produced a supernatant factor thought to come from T cells, and thus was called T cell factor (TCF). This TCF readily adoptively transferred CS and DTH to naive recipients and showed to have mast cell activating properties [16, 17, 29-31]. However, since proof was lacking that TCF was the product of T cells, the name was changed to PCl-F as the product of PCl contact immunization. PCl-F was found to be remarkably antigen (Ag) specific compared to another similar acting heterologous specific factor from immune cells of oxazolone sensitized mice (OX-F) [29]. PCl-F had Ag-specificity in ear swelling [29] and activation of mast cells, but presence of IgE antibody was ruled out as the cause [16, 31]. The fact that PCl-F had the specificity of an antibody led to examination of B cells as a source. It was subsequently demonstrated that the unusual B cell subset of B-1 B cells, that resides in the peritoneal cavity and is T cell independent in their activation and antibody production, was found to be activated very early (hours) after skin contact painting to immunize for CS. PCl-F is produced within hours of contact immunization by the activated B-1 B cells. Amongst other proteins, this PCL-F contained antigen-specific IgM [32-41]. In fact, monoclonal anti-TNP IgM myeloma proteins could substitute for PCl-F in reconstitution of CS in $JH^{-/-}$ pan immunoglobulin-deficient mice. Further research demonstrated that smaller components derived after fractionation of IgM were similarly able to reconstitute the initiation of CS. Thus, addition of an early-acting initiation component, like PCl-F or 1 day immune cells as a source of B-1 B cell IgM antibody, allowed the T cells to be recruited for full elicitation of CS. Not only pentameric IgM antibody was active in CS-elicitation, but also trimers, dimers, monomers, and heavy and light chain dimers that were not clearly known previously to be present in IgM preparations [19]. Although all of these fractions of IgM were active, it was shown that two mechanisms

were involved. The pentamer down to the heavy chains activated the mast cells indirectly. They were found to be complement dependent by forming immune complexes with circulating hapten self protein complexes generating C5a to activate the mast cells via their receptor for C5a [42-44]. In contrast and surprisingly, the Ag-specific polyclonal light chains plus antigen activated the mast cells directly in a manner analogous to IgE antibody. These findings corresponded with finding that light chains can activate mast cells [18, 19]. Indeed, an isolated immunoglobulin FLC of anti-TNP Ag specificity, could fully substitute for PCl-F in our system by reconstituting absent elicitation of CS in $JH^{-/-}$ mice that were unable to elicit CS without a CS-initiator like PCl-F.

Immunoglobulin free light chains mediate antigen-specific mast cell activation

As described above, the TCF consisted of a mix of several proteins. The presence of immunoglobulin light chains in this fraction was ultimately demonstrated after multiple steps of purification. Light chains are detectable with a size of 22-27 and 44-55 kD indicating that they are not covalently linked to heavy chains, but present in free form in a monomeric and dimeric conformation, respectively (unpublished data). To demonstrate that these “TCF components“ were critical for inducing the antigen-specific mast cell activation, hapten-specific FLCs were obtained by reducing and alkylating trinitrophenol (TNP)- and oxazolone-specific IgG_1. Subsequent intravenous injection of purified hapten-specific FLCs followed by a topical ear challenge with the appropriate hapten induced an increase in ear thickness as compared to unsensitized controls [18, 19]. This FLC-induced ear swelling response is dose-dependent and comparable to the responses elicited after sensitization with hapten-specific IgE. In addition, passive sensitization with culture supernatant from *in vitro* cultures of spleen and lymph nodes from sensitized mice (PCL-F) resulted in the transfer of hypersensitivity only when FLCs were not depleted using immobilized antibodies specific for κ-light chains. These data showed that active epicutaneous sensitization results in the production of hapten-specific FLCs, as early as one day after sensitization. Passive sensitization with antigen-specific FLC of pan B cell-deficient (μMT) followed by local challenge resulted in a significant ear swelling response. This finding indicates that the FLC-mediated reaction is not due to an interaction between FLCs and circulating immunoglobulins and therefore not dependent on intact immunoglobulins. Importantly, passive intravenous sensitization with hapten-specific heavy chains or non-specific light chains did not elicit an ear swelling response indicating that immunoglobulin heavy chains only weakly activate complement and do not activate mast cells.

Although controversial to some extent [45], it has been shown previously that the initiation of a profound delayed type contact hypersensitivity reaction is dependent on the activation of mast cells [23, 46-49]. This activation results in the immediate release of various mediators like the vasoactive serotonin (5-HT) and histamine, proteases, lipid-derived mediators (leukotrienes etc.), chemokines and cytokines (particularly TNF-α and MIP-2 [46]) leading to local expression of vascular activation markers (through TNF-α for example), vasodilatation and an increased vascular permeability (leading to the early and then late phase response) and attraction of immune cells [50]. Initiated by the early response, T cells are recruited locally and then the Ag-specific minority is activated in the tissues by Ag-MHC complexes on a few local antigen presenting cells, like dendritic cells. There, activated T cells produce various cytokines that induce the local tissue cells to produce chemokines that recruit a non-specific inflammatory cell infiltrate (mainly monocytes and neutrophils) from the vasculature, leading to the late phase response [50].

To test whether the FLC-mediated hypersensitivity was dependent on mast cells, mast cell-deficient ($WBB6F_1$-$Kit^{W/W-v}$) mice were passively sensitized with FLC. Subsequent challenge with the appropriate antigen did not result in a significant ear swelling response [18, 19]. Local reconstitution of bone marrow derived mast cells at the site of challenge 6 weeks before active sensitization and challenge fully restored the ear swelling response. Moreover, histological and ultrastructural analysis of mast cells at sites of challenged tissue showed marked signs of degranulation. Comparable changes in mast cell ultrastructure were observed earlier when analyzing the effects of TCF on mast cells [51]. Direct binding of FLCs to the mast cell (BMMCs) membrane was also shown using FITC-labeled FLC and beads coated with FLCs to visualize binding to mast cells. These data indicate that FLC-induced hypersensitivity is mediated via mast cells, yet the mechanism by which FLCs exert this function is still unclear and a specific FLC receptor has not yet been fully characterized. The involvement of the common γ-chain, involved in signal transduction after cross-linking of the IgE and IgG_1 high affinity receptors FcεRI and FcγRIII, respectively, is excluded since normal ear swelling responses were elicited in common γ chain-deficient mice ($FcR\gamma^{-/-}$) compared to wild type mice [18]. In addition, it was shown that the FLC-mediated contact sensitivity is independent of the action of complement [19].

F991, antagonist of FLC

Tamm-Horsfall protein (THP; uromodulin) is a naturally occurring protein that is highly expressed in the kidney and has the capacity to specifically bind FLCs [52]. THP is a heavily glycosylated, monomeric protein produced and secreted by cells of the thick ascending limb

of the loop of Henle. Analysis of the light chain-binding location site within THP revealed the amino acid sequence responsible for the FLC-THP interaction [53]. Using this amino acid sequence, a 9-mer peptide was tested for its ability to prevent FLC-binding to FLC targets, like a specific receptor on mast cells. Using this FLC-antagonist, F991, the capacity of FLCs to initiate contact sensitivity was further demonstrated, since intravenous injection of F991 prior to challenge resulted in complete inhibition of the early ear swelling response after both passive and active sensitization. The late phase response was inhibited as well in the active sensitization model [18]. F991 dose-dependently inhibited the contact antigen-induced ear swelling responses mediated by FLCs but in contrast, it was ineffective in inhibiting IgE-, IgG- and IgM-mediated responses.

FLC production and distribution

B cells are responsible for the production of immunoglobulins and are therefore critical in the humoral immune response. In addition, B cells play a critical role in host defense by initiating and/or modifying the immune response via antigen presentation to T cells and the production of multiple pro-inflammatory cytokines and chemokines. Intact immunoglobulins consist of two identical heavy chains and two identical light chains which are covalently linked in heavy and light chain pairs. Both heavy and light chains have a C-terminal constant region and an N-terminal variable region, the latter containing the antigen binding complementary determining regions. The variable regions of one disulfide bonded heavy and one light chain pair together constitute one of the antigen binding sites. The diversity of variable regions is maintained by random rearrangement of a multitude of Ig variable (V), diversity (D), and joining (J) gene segments for heavy chains and V and J segments for light chains. Furthermore, somatic hypermutation of variable regions occurs following antigen activation [54]. The C-terminal Fc region of the constant part of the heavy chains determines the (sub) class of the immunoglobulin (IgG, IgE, IgM, IgA, IgD), and thereby its biological function. Two types of light chains are produced by mammalian B cells, either kappa (κ) or lambda (λ) [55]. The ratio in which both types are produced varies significantly among species. In human for instance, half to two-thirds of the light chains produced is kappa, whereas mice predominantly produce kappa FLCs (~95%) [56, 57]. The production of heavy and light chains is independent of one another and normally there is a 10-40% overproduction of light chains compared to heavy chains [58-63]. The secretion of heavy chains is tightly regulated and will not occur as long as tetrameric antibodies are not formed. In this case, the free heavy chains are not transported to the Golgi complex and are degraded internally [64]. In contrast, B cells are able to secrete light chains that are not incorporated into immunoglobulins, leading to the presence of unbound FLCs in the extracellular environment and in serum. At this moment it

is unclear whether all activated B cells randomly overproduce FLCs, or whether this is somehow regulated. Possibly, the AID induced mutations in the V region and consequent affinity for antigen, the B cell type and/or strength of its activation signals, B cell microenvironment, and/or maturation stage may all influence FLC production [60, 65-68].

FLCs can be detected in serum, but also many other body fluids like urine, sputum and saliva, nasal secretions, bronchial alveolar lavage fluid, and synovial and cerebrospinal fluid. In all body fluids kappa and lambda FLCs predominantly exist as monomers (22-27 kD) and covalently and non-covalently bound dimers (44-55 kD), but also polymeric forms of kappa and lambda FLC occur. Concentrations of FLCs are low at birth and gradually increase by age until adulthood [69-72]. The 'normal' range of FLC concentrations in serum of healthy adults is between 3.3-19.4 mg/L for kappa and 5.7-26.3 mg/L for lambda, but can be highly dependent on the detection assay employed and calibration standards [56]. The kidney is the main organ responsible for the removal of FLCs from the circulation. After glomerular filtration, FLCs are primarily removed by proximal tubular epithelial cells in which lysosomal proteolysis of FLCs takes place. Constituent peptides and amino acids from FLCs are subsequently returned to the circulation. When FLC concentrations exceed the reabsorptive capacity of the proximal tubular epithelial cells, high amounts of FLCs will be excreted in the urine. The precise clearance of FLCs by the kidney is unknown, but based on size and cationic charge serum half-lives of kappa and lambda FLC of 2-4 and 3-6 hours are calculated, respectively [52]. In multiple myeloma, a tumour of antibody producing plasma cells, huge amounts of light chains are secreted in the urine and complex with Tamm-Horsfall protein to obstruct urine flow in the tubules to produce significant renal impairment; a condition known as myeloma kidney. In addition, patients suffering from other plasma cell dyscrasias which are associated with an increased FLC production show increased kidney injury [52].

Antigen binding by FLCs

In intact immunoglobulins the variable regions of both heavy and light chains together create two antigen binding sites per IgG and ten per IgM pentamer to mediate antigen binding. The overall binding of a complete single antibody is called affinity, which is determined by the variable regions in the immunoglobulin heavy and light chains. The presence of the variable region within light chains, together with the fact that many FLCs form dimers mimicking binding site of an intact antibody, indicates that FLCs may also have the capacity to bind antigen. However, studies concerning the binding of FLCs are contradicting and indicate that binding affinities of FLCs can be either negligible, equal, or even exceed those of parent

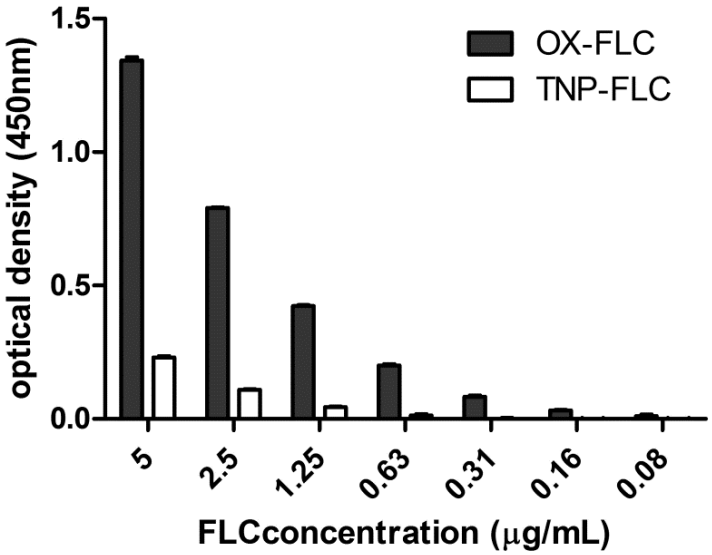

Figure 1. Antigen-specific binding of immunoglobulin free light chains (FLCs). Oxazolone-specific FLCs (OX-FLC) specifically bind to plate bound OX-conjugated albumin in a concentration dependent manner. Compared to OX-FLC, trinitrophenol-specific FLCs (TNP-FLC) hardly show binding to OX indicating an antigen-specific binding capacity. Background staining of FLCs to albumin is negligible (not shown). Based on these binding data we calculated the binding affinity (Km) of OX-FLC to OX-conjugated albumin using a Lineweaver Burke plot of 0.12μM which is relatively high.

immunoglobulins [73]. The *in vivo* data described above regarding the FLC-mediated mast cell activation indicate that FLCs are able to bind antigen by recognizing specific epitopes. Further, the fact that multiple FLCs on the surface of the mast cell are binding antigen means that their lower affinity still results, through multiple binding, into appreciable binding affinity resulting in significant Ag-specificity. Thus, CS-initiating ear swelling responses dependent on FLC bound to mast cells cannot be elicited by challenging the ears with an irrelevant hapten. Other preclinical models which will be discussed below, also suggest the presence of antigen-specific FLCs that mediate immunological responses by binding antigen specifically [74-77]. In line with these data, it is possible to demonstrate antigen-specific binding of oxazolone- (OX) and trinitrophenol- (TNP) specific light chains using western blot and ELISA techniques (Figure 1). At present, assays for detecting antigen-specific human FLCs are lacking [78]. Only one paper described the presence of *Toxoplasma gondii* specific FLCs in cerebrospinal fluid from AIDS patients suffering from encephalitis [67]. Technical limitations which, amongst others, together with a possible lower binding affinity of FLCs and the lack of positive reference controls, might hamper detection of specific FLC in body fluids. Moreover, in case of analyzing serum, antigen-specific FLC concentrations may be too low to detect due to the short half-life of FLCs in combination with a considerable time of obtaining the sample after antigenic stimulation. Analysis of body fluids that contain FLCs produced locally in affected tissues might improve detection as demonstrated in cerebrospinal fluid [67]. Other

interesting body fluids to analyze clinically are nasal secretions and synovial fluid, since highly increased FLC concentrations can be found in rhinitis and rheumatoid arthritis patients, respectively (see below) [79, 80].

Mast cell activation

Mast cells are dispersed throughout most tissues in the body but the majority of mast cells is located at anatomical sites that are in direct contact with the environment such as the skin, gastrointestinal tract, and the airways. This distribution provides them with the capability of initiating immune responses as soon as the body comes in contact with a pathogen [81, 82]. The mechanisms of activation of mast cells, mediator release, the effector functions of mast cells in different immune responses and its immunoregulatory functions are extensively reviewed previously but will briefly be addressed below. Next to FLC, multiple other signals including IgE, IgG, complement factors, cytokines, toll-like receptor ligands, bacteria, and viruses are able to activate mast cells under general or more strictly defined conditions [2, 83-85]. Three types of mediators released by activated mast cells are recognized: 1; pre-formed, granule-associated mediators like histamine, neutral proteases and heparin. 2; *de novo* synthesized lipid mediators like LTC4, LTB4 and PGD2. 3; various newly synthesized and secreted cytokines and chemokines [82, 83, 86]. Dependent on the types and strength of activating signals, mast cells can undergo complete or partial degranulation, also referred to as piecemeal degranulation. The former type of degranulation results in an excessive, more random release of prestored mediators, whereas piecemeal degranulation is the secretion of a selective subset of prestored mediators [85, 86]. The differential release of mediators by partial degranulation is again highly dependent on the type and strength of the stimuli. For instance, partial mast cell degranulation was documented on the basis of electron microscopy and differential release of mediators in allergic cutaneous hypersensitivity due to PCl-F [22-28]. Ultimately, not only the type and strength of the stimuli but also the type of mast cell itself will determine the mediators that will be released. Mast cells start maturation once they entered the tissue in which they will ultimately reside during their lifespan. Consequently this means that the anatomical site of maturation together with its (local) microenvironmental conditions may largely influence maturation (or alter phenotype after maturation) leading to phenotypically distinct subpopulations of mast cells, a phenomenon called mast cell heterogeneity.

Physiologically, mast cells are believed to be important for maintaining homeostasis. Mast cells essentially contribute to innate immune defences against parasites, bacteria, and to viral pathogens as well, and additionally mast cell are involved in modulating adaptive

immune responses and inducing tolerance [87-89]. Moreover, mast cells are shown to be crucial to wound healing processes, at least partially by initiating immune responses after activation by endogenous danger signals, such as IL-33 released from necrotic structural cells [90-92]. Despite these beneficial actions for the host, mast cells are highly associated with allergic disorders, including non-IgE mediated allergies [50, 93, 94]. In addition, mast cells are increasingly being recognized as a critical cell type involved in the initiation, modulation and/or progression of autoimmune diseases and cardiovascular disorders [9, 95-99]. The main autoimmune diseases that are currently being recognized to be significantly influenced by mast cell function are multiple sclerosis, rheumatoid arthritis, bullous pemphigoid, and insulin-dependent diabetes mellitus. Non-IgE-mediated activation mechanisms appear to be primarily important in autoimmune disorders since evidence of IgE-mediated mechanisms are limited, except for bullous pemphigoid, and some cases of Grave's ophthalmology and rheumatoid arthritis [100-103]. Tumourigenesis, the process of initiation and progression of tumours, is another disease type to be announced in which mast cells appear to have significant contribution to disease outcome [104-108]. In many human tumours the number of tumour infiltrating mast cells correlates with increased intratumour microvessel density, enhanced tumour growth and invasiveness, and poor clinical outcome. These clinical findings are supported by several rodent tumour models showing mast cells to be the first immune cell to enter the premalignant tissue and that mast cell stabilization or deficiency attenuated tumour growth [109-112]. However, a dual role is recognized for mast cells in tumour progression since its role in anti-tumoural immunity is also indicated in both clinical and preclinical studies [112-118]. Interestingly, the pro- or anti-tumourigenic effector function of mast cells seems to be determined by the microenvironment (i.e. peritumoural or intratumoural) and/or the presence of specific activating factors. This latter was shown by the tumour growth inhibitory effect of mast cell-derived IL-6 released upon TLR2 activation [112].

As briefly discussed above, mast cells are clearly associated with different disease pathologies but their definite functions are far from straightforward and probably depend on multiple factors. Different outcomes between laboratories investigating similar preclinical disease models in wild-type and mast cell-deficient mice more or less subscribe this by a lack of consistent proof for mast cell dependency in some disorders. The reason for this discrepancy might be due to microenvironmental differences or variation in sensitization/immunization protocols. Another factor contributing to differences in preclinical experimental outcome is the use of different mast cell-deficient mouse strains. Two commonly used mast cell-deficient strains, WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ and C57/BL6-*Kit*$^{W\text{-}sh/W\text{-}sh}$, contain a naturally occurring mutation in the c-kit receptor. Another mast cell-deficient strain is the WCB6F1-*Kitl*^{Sl}/*Kitl*$^{Sl\text{-}d}$, carrying spontaneous mutation in the c-kit ligand [119]. The drawback of

these strains however, is that they are not only deficient for mast cells but also display other hematopoietic and non-hematopoietic abnormalities. *Kit*$^{W\text{-}W\text{-}v}$ mice have abnormalities such as neutropenia, macrocytic anaemia, γδ T cell deficiency, and deficiencies in melanocytes, germ cells and interstitial cells of Cajal. On the other hand, the more recently characterized *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice have normal numbers of neutrophils, and are not anaemic and infertile, which makes them more attractive candidates for mast cell research. Interesting examples of preclinical models showing discrepant results on the involvement of mast cells in mediating pathology are models regarding delayed hypersensitivity as described above, but also rheumatoid arthritis (RA) and experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis. The critical role of mast cells in RA was shown in different RA models using *Kitl*^{Sl}/*Kitl*$^{Sl\text{-}d}$ and *Kit*$^{W/W\text{-}v}$ mice [120-122]. On the other hand, antibody-mediated arthritis and collagen-induced arthritis are not impaired in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice [121, 123]. In analogy to these findings, EAE models performed in wild-type and *Kit*$^{W/W\text{-}v}$, and also *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, suggested a detrimental role for mast cells in disease pathology [124, 125]. Yet, using *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice in the same experimental model showed that mast cells are dispensable for EAE development or even protective against EAE responses [126, 127]. This latter study also indicated an immunization dependent effect in the *Kit*$^{W/W\text{-}v}$ strain [126].

A promising technique that might overcome at least some of the problems caused by the limitations encountered in naturally occurring mast cell-deficient mice is the use of Cre/*LoxP* technology. This technology allows the generation of mast cell-deficient mice without substantially affecting other cell types. In addition, mast cell-specific gene expression of certain mediators can be knocked out which allows to investigate the contribution of mast cell-derived cytokines or chemokines for example, which can also be produced by other immune cells [1, 128].

Other effects of FLCs

FLCs have been shown to display other biological activities besides their involvement in antigen-specific mast cell activation. Proteolytic activity for different substrates has been shown for several monoclonal FLCs and for recombinant light chain variable fragments [129, 130]. A study by Sun *et al.* demonstrated that the immune system can generate FLCs containing catalytic sites in response to polypeptide antigens enabling FLCs to hydrolyze proteins in an antigen-specific manner [131]. Another study showed that lambda FLC dimers, not control kappa or lambda light chains, contributed to the initiation of the alternative complement pathway activation in a patient suffering from membranoproliferative

glomerulonephritis [132]. Yet, it is unknown whether this lambda FLC capacity can be found in other patients/conditions as well.

Other functions of FLCs are based on their ability to bind other cells than mast cells, such as neuronal cells and macrophages. Antigen-specific FLCs can bind to, and activate, murine dorsal root ganglion neurons upon application of appropriate antigen as measured by increases in intracellular Ca^{2+} [133]. This might indicate that FLCs are involved in local neurogenic inflammation and centrally-mediated sensations such as pain. Binding of FLCs to macrophages is suggested by early studies on TCF. TCF was shown to bind to macrophages which subsequently resulted in the rapid release of TNF-α, IL-1, IL-6, PGE2, and nitric oxide (NO) [134-136]. In contrast to FLC-mediated mast cell mediator release, these TCF-primed macrophages produced these mediators in the absence of a cross-linking reagent. Whether FLCs were the actual factors present in the TCF fraction responsible for these effects on macrophages is unclear. Non-antigen-specific FLCs also influence some essential neutrophil functions as it was demonstrated that FLCs significantly inhibited chemotactic neutrophil movement, reduced the activation of neutrophils, and attenuated neutrophil apoptosis [137, 138]. To what extent these biological actions of FLCs contribute to the development of an immune response or pathological conditions warrants further study. Additionally, basophils are other interesting cells to investigate as putative target for FLC-mediated responses and their involvement in allergy and immunoregulation [139, 140].

Aim and outline of this thesis

Data described so far point to an interesting inflammatory mediating function of FLCs. As described in this chapter however, most evidence is based on experiments performed in preclinical mouse models. The focus of this thesis is to gain more insight in the presence, distribution, and putative functional role of FLCs in different human disorders.

Allergy is a disorder which is of major interest in our current research. Binding of IgE to its high affinity receptor (FcεRI) and subsequent cross-linking of receptor-bound IgE is considered a key contributing factor to most types of allergies. However, depending on the type of allergy, a subgroup of patients displays common allergic symptoms whereas antigen-specific IgE cannot be detected. The role of IgE- and non-IgE-mediated mechanisms in allergic disorders is reviewed in **chapter 2**, and the possible role of FLCs in the pathogenesis of non-IgE-mediated allergies is discussed. Subsequently, the presence of FLCs was analyzed in allergic rhinitis and nonallergic rhinitis with eosinophilic syndrome (NARES) patients (**chapter 3**), chronic rhinosinusitis with and without nasal polyposis (**chapter 4**), and hypersensitivity pneumonitis and idiopathic pulmonary fibrosis (**chapter 5**).

Besides the presence of FLCs, other inflammatory markers such as IgE, IgG, and tryptase (marker for mast cell activation), and presence of B cells, plasma cells and mast cells were analyzed in affected tissues. Besides allergy, several other inflammatory disorders display features of mast cell activation without a clear known activating factor. Rheumatoid arthritis is one of these disorders, and evidence for a possible involvement of FLCs in this disorder is described in **chapter 6**. Another pathology increasingly associated with inflammation and mast cell involvement nowadays is cancer which is reviewed in **chapter 7**. FLCs appeared to be abundantly present within human cancer tissues of different organs and of different aetiology, and showed to be associated with poor clinical outcome in breast cancer patients (**chapter 8**). Furthermore, in a preclinical melanoma model it was shown that tumour growth was highly dependent on the presence of both FLCs and mast cells.

FLCs can also bind to and influence cell function of cells other than mast cells. However, FLC-mediated effects on many inflammatory cells are still not analyzed. In **chapter 9**, it is demonstrated that FLCs bind to human basophils and that cross-linking of FLCs modulates IgE-mediated basophil activation. Another scarcely described aspect of FLCs is the existence of a possible regulatory mechanism involved in controlling its secretion as briefly discussed above. **Chapter 10** describes an unexpected finding that FLC monomers and dimers appear to be secreted as distinct entities and that FLC dimers have a significantly higher antigen-specific binding affinity compared to FLC monomers In **chapter 11**, the findings described in this thesis are summarized and discussed considering earlier work as described in this chapter and other recent developments in the field of immunology.

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Atopic and non-atopic allergic disorders: current insights into the possible involvement of free immunoglobulin light chains

This chapter is published in *Clinical and Experimental Allergy*, 2009;39:33-42

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Abstract

Allergic diseases have become a serious global health problem in the developed world. IgE interacting with its high affinity receptor FcεRI is considered a major contributing factor to most types of allergies, but depending on the type of allergy however, a subgroup of patients displays common symptoms yet lack elevated levels of total serum IgE and/or antigen-specific IgE. Novel therapeutic strategies such as anti-IgE therapy may therefore not be applicable to these patients. It is clear however that these patients do display activation of mast cells. In several patients suffering from immunological disorders, an increase in immunoglobulin free light chain levels can be detected. Previously, we have described the capability of free light chains to elicit immediate hypersensitivity responses. In this review, we will discuss the role of IgE and non-IgE mediated mechanisms in allergic disorders and point out a possible role of free immunoglobulin light chains in the pathogenesis of the non-atopic types of these allergies.

Introduction

Despite advances in general healthcare over the last five decennia, the occurrence of immunological disorders have risen in industrialized countries [1]. This rise may be related to an improved standard of hygiene, leading to a decreased microbial exposure of the developing immune system at early life. This hypothesis is currently subject of many studies and is heavily discussed in scientific literature [2, 3]. Traditionally, allergies are treated with anti-histamines, corticosteroids and leukotriene receptor antagonists, but now many novel therapeutics are in clinical development which may change future treatment regimens. In cases in which the allergen is known, avoidance to encounter the allergen or specific immunotherapy may reduce the induction of allergic symptoms. Thus far, allergen immunotherapy is the only current method that has long-term benefits in the reduction of allergic rhinitis and asthma [1].

Central in an allergic disorder remains activation of mast cells [4, 5] through various pathways. Activation through IgE is amongst the best described and the role of IgE and its high affinity receptor FcεRI is undisputed [6, 7]. Increased levels of IgE have been detected in patients with food, skin, airway and drug allergies. Crosslinking of IgE bound to its high affinity receptor FcεRI causes activation and degranulation of mast cells. The subsequent release of pro-inflammatory mediators is key to the onset of several physiological and immunological events.

Allergic disorders displaying increased levels of total IgE, and antigen-specific IgE are known as atopic immunological disorders. Beside these disorders, a significant number of cases display comparable symptoms to their atopic variants, yet lack increased serum IgE levels and/or antigen-specific IgE. These cases are defined as non-atopic. Although the majority of allergic disorders may be classified as atopic, in some cases the percentage of non-atopic patients remains quite large. For example, about 40 percent of cow's milk allergic (CMA) patients does not display increased levels of IgE yet suffer in equal extent as their atopic counterpart. Besides food allergies, dermatitis, asthma and rhinitis also display a considerable proportion of cases in which IgE cannot be identified as the mediator for initiation of the immune response. Mast cells can also be activated by various specific and non-specific mechanisms e.g. complement, neuropeptides, superantigens and cytokines (reviewed in [5]). Ag-specific activation may also occur via crosslinking of the FcγR receptor family [8], presently insufficient data exist to relate non-atopic allergic responses to the presence of allergen-specific IgG's.

In this review, we will focus on the differences and similarities between the pathophysiology of atopic and non-atopic forms of food allergy, dermatitis, asthma and rhinitis. In addition, we

will discuss the possible involvement of free immunoglobulin (IG) light chains in these disorders.

Food allergy

Food allergy is next to hay fever and allergic asthma one of the most common allergies [9]. Numerous cases of food allergy show a wide variety of antigens capable of eliciting an allergic response. Amongst the most prevalent are milk, peanut, shellfish and soybean containing products responsible for over 90% of all cases. Fortunately, most forms of food allergy are no longer present when becoming an adult and therefore the prevalence of food allergies is highest among infants [10]. Despite the high incidence in infants, the exact mechanism by which a person becomes allergic to a food-related allergen remains unclear. High levels of IgE measured in serum of food allergic patients suggest a critical role for IgE. In cow's milk allergy, however, only 60% of the patients display increased levels of IgE yet show signs of mast cell activation [11].

During the normal development of the gastrointestinal system, the epithelial barrier separating the lumen from the mast cells residing in the sub-epithelial layer provides sufficient protection to prevent sensitization and challenge. In some cases however, this barrier is not fully closed and potential allergens can pass through and cause activation of the mast cells. Inflammation in the gut often coincides with increased levels of various lymphocytes and eosinophils. IL-5 has been shown to be crucial for the development of eosinophilic esophagitis and the use of anti-IL5 based drugs (mepolizumab) shows favourable results. Whether this therapy may be applicable to a broader group of food allergic patients remains to be investigated. Recently, the role of IL-9 and mast-cell mediated gut leakiness responsible for oral antigen sensitivity in mice has been elucidated [12]. Overexpression of IL-9 in the intestine resulted in an increased susceptibility to oral antigen-induced intestinal anaphylaxis. Moreover, addition of cromolyn, a mast cell stabilizing agent, prevented sensitization supporting evidence that mast cells play a crucial role in food allergy. These findings illustrate both the importance of IL-9 and mast cells in gastrointestinal allergic disorders.

In order to determine the allergen(s) to which the patient is allergic, the use of skin prick tests in combination with measurement of specific-IgE serum levels have proven to be a valuable tool. This diagnostic strategy is however less applicable in non-atopic patients in which it may take days to develop a response to a certain allergen. In addition, non-IgE mediated hypersensitivity to food allergens is not confined to only one allergen but to several allergens [13]. Although the exact mechanism causing allergic responses to these food

allergens remains unclear, the presence of an antigen specific-based mechanism is most likely since dietary restriction by these patients shows a decrease in allergic response.

Dermatitis

The prevalence of atopic dermatitis (AD) has risen over the last 2 decades. AD compromises various skin abnormalities such as atopic eczema which now affects over 25% of children and 3% adults in the industrialised nations [14] despite advances in therapeutic interventions [15].

Numerous studies have implicated a role for mast cells in development of AD. Especially the use of mast cell deficient mice (W/W^v) [16] has clearly identified mast cells to be key effector cells in acute and chronic AD [17]. Also, the presence of elevated levels of mediators most likely released by mast cells has been detected in skin samples of AD patients. Skin samples taken from AD patients indeed display increased levels of histamine and IL-13 [18, 19]. Corresponding to this, acute AD patients show a normal amount of activated (degranulated) mast cells whereas chronic AD patients display an increased number of mast cells in skin lesions.

Histological investigation of AD lesions shows both CD4+ and CD8+ T cells concurrent with the presence of IL-4, IL-5 and IL-13, which is produced by these cutaneous T cells. Activation of these T cells may be explained by the increased presence of antigen presenting cells. Increased expression of FcεRI on antigen presenting cells like Langerhans cells and epidermal dendritic cells could facilitate antigen presentation [20].

Elevated serum levels of IgE have recently been questioned to be a correct parameter for diagnosis of AD. Although anti-IgE therapy seems beneficial to patients with high serum IgE titres suffering from severe atopic dermatitis (communicated by Ring *et al*, CIA meeting 2008), such treatment would likely be less successful in patients with normal serum IgE concentrations which appropriates between 16% and 45% of AD patients [21]. Notwithstanding the normal IgE levels, non-atopics still suffer from comparable allergic symptoms (e.g. skin lesions) as atopic patients [22]. Parallel to these normal IgE levels, the expression of the high affinity IgE receptor (FcεRI) on mast cells and Langerhans cells is also decreased. It remains unclear whether these patients have developed a complete non-IgE mediated hypersensitivity or “switched” from an IgE mediated process to a non-IgE mediated mechanism. In a recently developed mouse model for atopic dermatitis, Chan and coworkers were able to induce atopic dermatitis comparable to human conditions. Crossbreeding IL-4 Tg mice with SKH1 hairless mice resulted in the elicitation of symptoms with histological, immunological and clinical similarities to human dermatitis. Interestingly,

these mice did not show increased IgE and IgG1 serum levels. This mouse model, since it is lacking the dense fur, is a promising tool to study non-atopic dermatitis comparable to human conditions [23]. Patients without increased levels of IgE are also defined as atopiform dermatitis (AFD) with a female predominance for non-IgE mediated AD/AFD [24, 25]. Interestingly, idiopathic anaphylaxis, for example elicited by peanuts, is also more common in women comprising 65% of all idiopathic patients [26, 27]. Whether there is a common mechanism between both disorders remains unclear.

Asthma

Asthma is a relatively common disorder of the lower central airways, associated with a wide range of symptoms including wheezing, cough, chest tightness and shortage of breath. It is a chronic inflammatory disease in which many cell types and cellular elements play a role [28]. This can result in reversible and variable bronchial smooth muscle spasm, hyperreactivity, and airway obstruction. Ultimately, chronic inflammation can lead to irreversible airway remodelling which appears to play a role in airway dysfunction in asthma [29]. Because the pathogenic mechanisms of asthma are still not clear, the definition of asthma includes different phenotypes that are recognized on clinical grounds, and on the basis of genetic and causative pathological features [30, 31].

Atopic and non-atopic asthma are probably the most common phenotypes. Atopic asthma refers to a pathophysiology involving specific IgE-mediated tissue sensitization with a T helper 2 response, eosinophilia and increased numbers of activated mast cells [32, 33]. The underlying mechanisms leading to non-atopic asthma are less clear, but patients do not show increased levels of IgE or the presence of specific IgE, as evidenced by a negative skin prick test for allergens known to be associated with asthma. Although some authors report that 10 to 33% of total asthmatics are non-atopic [20, 34], other population-based studies showed that the proportion of asthma cases that are attributable to atopy may be less than 50% [35, 36]. A study by Pearce *et al.* found only a weak and inconsistent association between the prevalence of asthma and the prevalence of atopy with standardized comparisons across populations or time periods [35]. These data indicate that the role of atopy in the development of asthma may have been over-emphasized [37], and that factors other than IgE might be involved in the pathogenesis of a substantial group non-atopic asthma patients [38] and possibly atopic asthmatics as well.

Many immunological similarities have been reported between the atopic and non-atopic forms of asthma, but also some differences have been reported [39-45]. The cellular pattern of inflammation in atopic and non-atopic patients with asthma is characterized by a

increased numbers of eosinophils, mast cells, and T lymphocytes, but differences in the absolute numbers of infiltrating cells may exist between atopic and non-atopic asthmatics. In addition, non-atopic asthma is accompanied with increased numbers of neutrophils, which may not clearly be evident in atopic asthmatics [45, 46]. The subepithelial tenascin- and laminin-positive layers are significantly thicker in atopic asthmatics than in non-atopic asthmatics and healthy controls subjects [45]. In other studies, the only difference that could be found between the two forms of asthma was an increased number of macrophages in non-atopics [39, 47]. Mast cells appear to be important in both forms of asthma, and infiltration of airway smooth muscle by these cells is associated with airway dysfunction [48]. The expression of several cytokines in bronchial biopsy specimens and sputum appears to differ between both groups [45, 49, 50], although not all studies are in agreement with this [40, 41, 44]. More studies are needed to clarify the immunological differences in detail between the two forms of asthma. The clinical expression of atopic and non-atopic asthma, on the other hand, is highly distinct [51]. Non-atopic asthma appears to be more severe than atopic asthma and higher age and female sex are associated with an increased risk of non-atopic asthma compared to atopic asthma.

These differences found between the atopic and non-atopic form of asthma might support a difference in pathophysiology. Considering the similarities however, at least some overlap in the involved working mechanisms is likely. One of the putative working mechanisms in non-atopic asthma is the local production of IgE. Immunopathological comparisons of bronchial biopsies from atopic and non-atopic patients with asthma demonstrated similarities in expression of factors responsible for IgE synthesis. Levels of IL-4 and IL-13, which are important for IgE class switching, both seem to be increased in asthmatics independent of atopic status [40, 41], and significantly increased numbers of FcεRI-bearing cells are identified in both groups [39], which suggests the presence of IgE, since IgE regulates expression of its own receptor [52]. Moreover, local expression of germline transcript (Iε) and the ε heavy chain of IgE (Cε) and local class switch recombination to IgE are demonstrated in the bronchial mucosa [53, 54]. These data might suggest a local form of atopy in non-atopic asthmatics, referred to as ‘entopy’ by Powe *et al.* [55]. This ‘entopy’ could be responsible for the pathophysiology of atopic asthma as well, at least partly. Although this proposed mechanism of local IgE production might be relevant to both atopic and non-atopic asthmatics, a pathophysiology solely based on IgE may probably be too straightforward. The use of the recombinant humanized monoclonal antibody against IgE (omalizumab), for example, greatly reduces circulating concentrations of IgE in asthma patients. Although beneficial effects of omalizumab on asthmatic responses have been shown and discussed [56-58], it is also argued that most significant effects of anti-IgE treatment are marginal

reductions in airway inflammation and in exacerbation rate, whereas airway obstruction and hyperresponsiveness do not change significantly [59, 60]. Moreover, inflammatory reactions similar to those elicited after IgE-mediated mast cell activation can occur in the absence of IgE or expression of functional FcR on mast cells [61-65]. Although insufficient tissue penetration of a large molecule as Xolair could be responsible for the lack of efficacy in some cases, these findings imply that (an)other mechanism(s) could play a role in the pathophysiology of atopic and non-atopic asthma .

Rhinitis

Rhinitis is defined as an inflammation of the nasal mucosa and is characterized by nasal symptoms including rhinorrhea, sneezing, nasal blockage and/ or itching of the nose [66]. Although rhinitis and asthma are common co-morbidities, suggesting a concept of “one airway, one disease” [67, 68], we will discuss rhinitis separately from asthma.

Rhinitis can roughly be classified into infectious, atopic (allergic) and non-allergic/non-infectious [69, 70]. Atopic rhinitis has a Th2 inflammatory pathophysiology and is characterized by high (specific) IgE levels and infiltration of the nasal mucosa with basophils, Langerhans cells, eosinophils and mast cells [69]. In contrast, rhinitis is non-allergic/non-infectious when (atopic) allergy has not been proven by proper allergy examination (history, skin prick testing, measurement of serum specific IgE) and when infection can be excluded (nasal discharge is clear, watery, and not purulent). Diagnosis is usually made by process of exclusion. Few studies are performed with respect to non-allergic/non-infectious rhinitis and so it is still a poorly defined disease. Consequently, the pathophysiology is largely unknown and this disease is highly heterogeneous [71]. This means that this patient group is probably a mixture of patients suffering from nasal complaints, caused by different pathogeneses [71]. Aetiological mechanisms have been identified for some forms of non-allergic/non-infectious rhinitis (e.g. hormonal, gustatory), but for many other forms the cause is still unknown. This last group is also defined as idiopathic rhinitis (IR), which by definition, does not exhibit systemic atopy and lack an IgE- mediated aetiology [71]. About 25% of rhinitis patients have been reported to have non-allergic/non-infectious rhinitis [72-75], but also in perennial rhinitics an equal division between allergic and non-allergic patients has been reported [76]. This latter finding is supported by Zacharasiewicz *et al.* who showed that the proportion of rhinitis cases that is attributable to atopy is 53%, indicating that the connection between atopy and rhinitis symptoms is far from straightforward [77]. It further indicates that IR is a common disorder, in which a non-IgE mediated disease mechanism might be involved.

Many IR patients present nasal symptoms and nasal hyperreactivity that mimic atopic rhinitis [70, 74]. However, in what respect there are immunological similarities between atopic and idiopathic rhinitis is largely unknown. Studies on IR patients are limited, mainly because the large heterogeneity of this group complicates selection of patients and comparison of data. Nevertheless, Powe *et al.* showed that both atopic and idiopathic rhinitis patients had significantly more nasal mucosal mast cells and eosinophils compared with a normal control group [78, 79]. This contrasts with studies which did not find any significant differences in nasal mucosal cell infiltrates between IR patients and a control group [80, 81].

There are several mechanisms that might be involved in the pathophysiology of IR [70, 71, 76]. For example, autonomic neuronal dysfunction, over-expression of tachykinin response, dysfunction of the neuroendocrine system, mechanical stimuli, eicosanoids and localized mucosal inflammation possibly play a role in the pathogenesis of IR [71]. Perhaps, these different mechanisms can be involved in distinct disorders, which are all referred to as idiopathic rhinitis. A significant proportion of IR patients has positive responses to nasal challenges, suggesting that localized inflammation may be important in the absence of systemic atopic markers [82]. Furthermore, a subgroup of these patients with non allergic rhinitis with eosinophilic syndrome (NARES) usually respond to intranasal glucocorticosteroids [76, 83], which further suggests a role for inflammation in at least a part of the IR patients. A possible explanation for nasal allergic responses could be the local synthesis of IgE [84, 85]. Increased numbers of IgE+ cells in the nasal mucosa of both groups are found [78], ex vivo stimulation of nasal biopsies from allergic subjects with allergen increases IgE transcription [86] and the presence of specific IgE antibodies in nasal secretions was demonstrated in skin test-negative individuals [87].

Preclinical and clinical studies supporting a possible involvement of free IG light chains in non-atopic allergic disorders

In the past years, our group described the role of free IG light chains in the development of hypersensitivity-like responses [88-90]. In parallel to complete antibody production, a surplus of free IG light chains is generated and secreted by plasma cells [91-93] (Fig.1). Free IG light chains convey various biological activities [94]. In previous years, our group has demonstrated that free IG light chains can bind to mast cells and sensitize them for activation (Fig. 2). Upon crosslinking of surface-bound free IG light chain with cognate antigen, mast cells are activated to release their mediators (Fig.1). Experiments in mast cell-deficient mice have clearly identified mast cells to be crucial targets in free IG light chain-mediated hypersensitivity responses. F991, 9-mer peptide (acetyl-AHWSGHCCL-amide)

based on the free IG light chain binding sequence of Tamm Horsfall protein [95], was identified as an specific antagonist for the immunological actions of free IG light chains [88]. Administration of F991 completely blocks hypersensitivity responses elicited by free IG light chains, but does not interfere with those induced by IgE and IgG_1 [88]. The efficacy of F991 is evaluated in both preclinical and clinical settings to further delineate the significance of free IG light chains in inflammatory diseases (see below). The possible role of free IG light chains in (non-atopic) allergic disorders as an alternative to IgE (Fig. 1 and Table 1) will be further outlined in the next paragraphs.

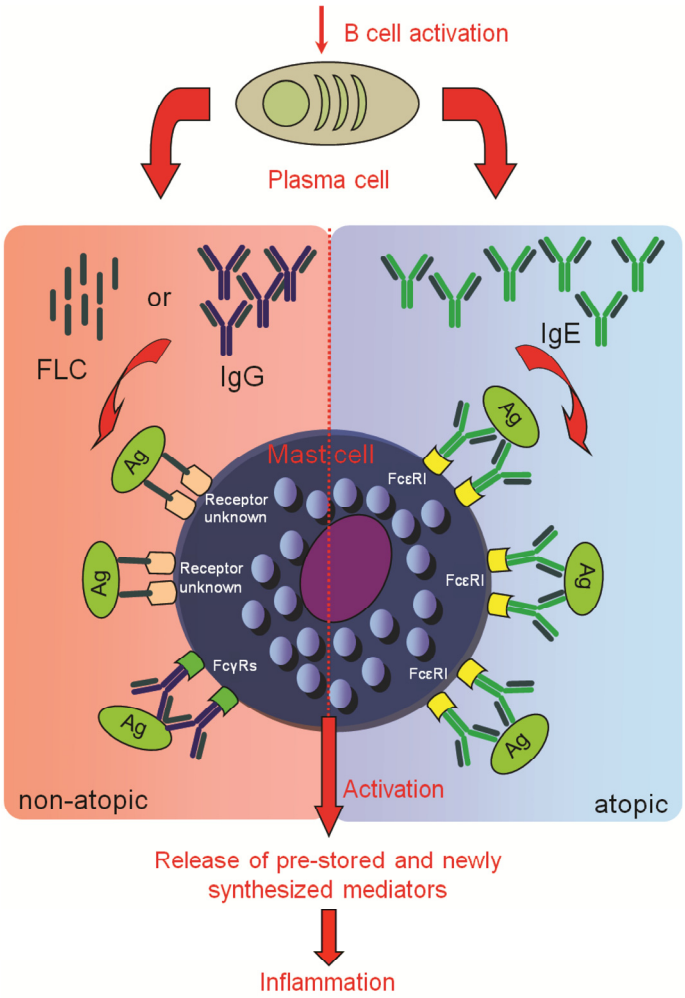

Figure 1. Distinct mechanisms are possibly involved in the pathogenesis of atopic and non-atopic disorders. Upon activation by antigen (Ag) and under the influence of specific costimuli, B cells will be activated to become antibody-secreting plasma cells. Antigen-specific complete immune-globulins, e.g. IgE and IgG, but also free IG light chains (FLC) are secreted in serum. IgE, IgG and free IG light chains sensitize mast cells through binding to specific receptors. Upon subsequent encounter with cognate antigen, crosslinking of surface-bound Igs or free IG light chains activates mast cells to release pre-stored and/or newly synthesized mediators. These mast cell mediators subsequently initiate and regulate inflammatory responses at the side of antigen encounter. IgE is the prime immunoglobulin produced in atopic disorders (right side of scheme), whereas non-atopic diseases are -by definition- non-IgE mediated. The involvement of free IG light chains is a potential mechanism regulating non-atopic disorders (left side of scheme). In addition, IgG could activate mast cells via FcγRI (human) or FcγRIII (mouse) receptors (left side of scheme).

Table 1. Comparison between general characteristics of IgE and immunoglobulin free light chain

Feature	IgE	Immunoglobulin free light chain
Normal serum concentration	~150 µg/L	κ: 3.3-19.4 mg/L [a] λ: 5.7-26.3 mg/L [a]
Serum half-life	1-2 days	2-3 h
Receptor	FcεRI and FcεRII	Unknown (γ-chain independent)
Target cell	Mast cell, basophil, Langerhans cell	Mast cell, others unknown
Immunological response	Type I hypersensitivity, Antigen presentation	Immediate hypersensitivity-like, Type IV hypersensitivity
Increased synthesis	Worm infection, Atopic allergic diseases	Non-atopic allergic diseases, Autoimmune diseases [b]
Therapeutic intervention	Immunotherapy, Anti-IgE monoclonal antibody	F991 [c]

[a] 95% reference intervals for serum concentrations of free immunoglobulin light chains when assayed with FreeliteTM [105].
[b]The clinical relevance of free light chains in human autoimmune diseases remains to be investigated.
[c] F991 is a peptide antagonist of free light chains that does not interfere with intact immunoglobulins, and can be administered by various routes [88-90]. The therapeutic efficacy of F991 remains to be investigated.
(Table adapted from [94])

Food allergy and free IG light chains

CMA patients react to specific proteins present in milk, but about 40% of these patients cannot be associated with increased serum titers of total or specific IgE. In mouse models for cow's milk allergy it is found that depending on the food allergen used either increased IgE or free IG light chain levels are found [96]; Schouten et al., submitted). Nevertheless, mice displayed similar allergic symptoms providing evidence that free IG light chains could act as an alternative to IgE to elicit these responses. In a mouse model for inflammatory bowel disease (IBD), it was shown that administration of the free IG light chain antagonist F991 resulted in a reduced development of diarrhea, infiltration of the colon by inflammatory cells, and mast cell activation (Rijnierse et al, submitted) and [97]. Although IBD is not directly comparable with food allergy [98], the results mentioned above clearly exemplify that free IG light chains could play a role in gastrointestinal disorders of various kinds.

Dermatitis and free IG light chains

In previous studies, we have shown that trinitrophenyl (TNP)-specific free light chains are produced when naive mice are exposed to TNP through cutaneous application. Reversibly, sensitization with TNP-specific free IG light chains causes a cutaneous type I

hypersensitivity-like response. Contact sensitivity responses to contact allergens can be abolished by prophylactic or therapeutic administration of the free IG light chain antagonist F991 [88, 89]. Although it remains crucial to measure free IG light chain levels in AD patients both locally and in serum, free light chains seem to be effective facilitators to invoke inflammation in the skin. Interestingly, in a recent study, significantly increased free kappa and lambda light chains concentrations were found in serum samples from children (until 3 years of age) with a severe form of AD when compared with healthy age matched controls [99].

Asthma/rhinitis and free IG light chain

Using a murine experimental model of non-atopic asthma our group has shown that the free IG light chain antagonist F991 abrogates the development of airway obstruction, hyperresponsiveness and pulmonary inflammation [62]. Upon administration of F991 prior to antigen challenge, mast cell activation could be completely inhibited. Additionally, F991 inhibited the neutrophil infiltration into the BAL fluid and inhibited development of tracheal hyperreactivity. Serum analysis of free IG light chains in adult asthmatic patients demonstrated of these are significantly increased in both atopic and non-atopic asthma [62].

In an ongoing study, we also found evidence for the presence of free IG light chains in atopic and non-atopic upper airway inflammation. Both kappa and lambda free IG light chain concentrations are highly increased in nasal secretions of atopic and non-atopic rhinitis patients compared to healthy controls. Moreover, concentrations were significantly higher in non-atopic patients compared to atopic patients. Histological examination showed that free IG light chains are associated with mast cells in nasal mucosa, supporting the hypothesis that free IG light chains may be involved in eliciting upper airway inflammation in rhinitis patients (Powe *et al*, submitted).

Clinical studies

Although studies in different murine disease models suggest an important role for free IG light chains in inflammatory diseases, no direct evidence on the (patho)physiological importance of free IG light chain in human disease is available.

A study to gain insight in the clinical efficacy of F991 [88] and the role free IG light chains in human allergic disorders has been carried out in house dust mite (HDM)- and peanut (PN)-allergic patients. An earlier phase I clinical study showed that F991 has no adverse effects in healthy human subjects when administered intravenously. In this follow-up study, the inhibition of allergic skin reactions was investigated after prophylactic local administration of

F991 in HDM- and PN-allergic patients. Wheal size and corresponding erythema were measured 15 minutes and 6 hours after injection of allergen extract to assess the effect of F991 on early- and late-phase skin reactions. Interpretation of the results of the study was greatly complicated by the fact that a number of patients exhibited a significant "irritant" effect to F991 injection frequently as large as the allergen-induced allergic reactions. However, when data were corrected for this F991-induced irritant reaction, a significantly smaller early-phase skin reaction after treatment with F991 compared to placebo was found in house dust mite-allergic patients (Table 2, unpublished results) suggesting a clinical efficacy of F991 in the treatment of these allergies. Yet, because of the irritant effect of F991 and the relative small sample size of the allergic groups (HDM, n=24; PN, n=15) in this study, further proof-of-concept studies using other routes of administration are warranted to ascertain the role of free IG light chain and the clinical efficacy of F991 in these allergic disease.

Table 2. Inhibitory effect of F991 on erythema and wheal reactions in house dust mite- and peanut-allergic patients 15 min after allergen challenge

	HEICWS*	**MWD***
House dust mite-allergic patients		
Erythema	42.1% (P=0.015)	38.1% (P=0.034)
Wheal reaction	24.4% (P=0.026)	16.3% (P=0.103)
Peanut-allergic patients		
Erythema	86.4% (P=0.354.)	83.4% (P=0.039)
Wheal reaction	30.9% (P=0.139)	27.8% (P=0.248)

* Percentages represent mean reductions in symptoms in the F991-treated group compared to the placebo-treated control group 15 minutes after allergen challenge. Data are corrected for the F991-induced irritant reaction.
HEICWS, Histamine Equivalent Intracutaneous Wheal Size; MWD, Mean Wheal Diameter.

Free IG light chains as a biomarker

The measurement of total and antigen-specific free light chains might be of primary interest and could possibly be useful as a diagnostic marker in allergic diseases. Total serum free light chains concentrations are considered prognostic markers in e.g. rheumatoid arthritis and multiple sclerosis [100-102]. Nakano *et al*. were unsuccessful in the measurement of specific free IG light chains as a diagnostic marker for patients with allergy. An important limitation of this study however was that analysis was carried out only with approximately 2% of total free IG light chains present in serum [103]. Presently, we are evaluating antigen-

specific free IG light chains in serum to determine whether measurement of antigen-specific free IG light chains may be useful as a diagnostic marker for allergic diseases.

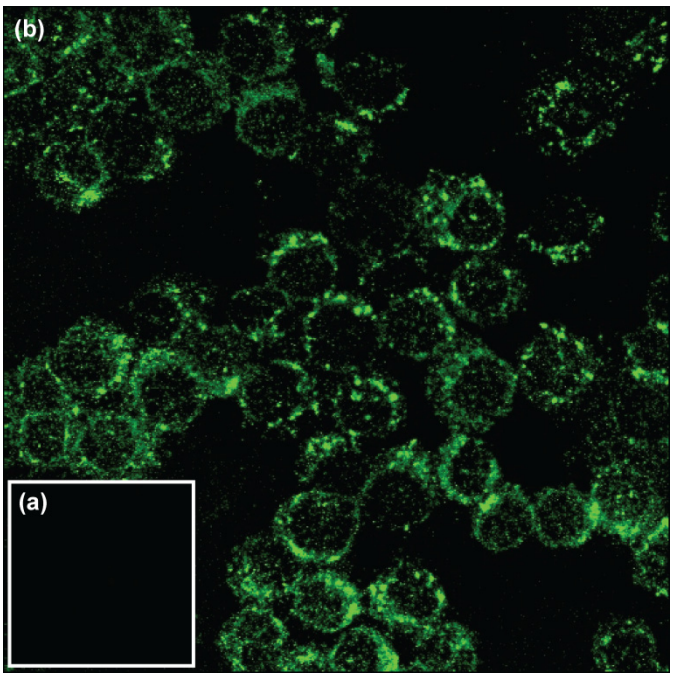

Figure 2. Confocal image of binding of free IG light chains to mast cells. Bone marrow derived mast cells from mice were incubated with FITC-labeled free IG light chain (**panel b**). To enhance fluorescence, all cells were subsequently incubated with an Alexa488 conjugated anti-fluorescein antibody. The intensity of the fluorescent signal suggests a patchy distribution of free IG light chain-binding proteins (receptors) on the mast cell membrane. Such clustered distribution has also been demonstrated earlier for FcεRI. **Panel a** displays unstained cells (labeling with only secondary antibody).

Conclusion

In this review, we have focused on the differences and similarities between the atopic and non-atopic allergy. We hypothesize that based on preclinical and clinical studies, immunoglobulin free light chains may be involved in the pathophysiology of different allergic disorders. In this respect, the most interesting groups of patients may be those which do not show increased levels of total serum IgE or the presence of antigen-specific IgE, but display local or systemic increases in immunoglobulin free light chains concentrations. Future studies are necessary to further delineate the importance of free IG light chains in allergic and other chronic inflammatory disorders [94, 104]. The immunoglobulin free light chain antagonist F991 may be an interesting candidate compound to therapeutically interfere in both preclinical and clinical settings to further delineate the significance of free IG light chain in inflammatory diseases.

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3

Evidence for the involvement of free light chain immunoglobulins in allergic and nonallergic rhinitis

This chapter is published in *Journal of Allergy and Clinical Immunology*, 2010;125(1):139-45, el-3

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Abstract

Background: Allergic rhinitis is characterized by mast cell degranulation induced by antigen cross-linking of IgE. It has been proposed that some patients with rhinitis show nasal allergy in the absence of systemic markers of atopy, termed *entopy*. Recent murine studies suggest the existence of an IgE-independent hypersensitivity response involving antigen-induced mast cell activation, mediated by immunoglobulin free light chains (FLCs).

Objectives: To determine whether FLC is associated with mast cell-mediated nasal hypersensitivity and its relationship with eosinophilic activity in allergic and nonatopic rhinitis.

Methods: Patients with allergic and nonallergic rhinitis with eosinophilia syndrome (NARES) had levels of soluble FLC measured in nasal secretions and serum. In addition, levels of the nasal inflammatory mediators mast cell tryptase and eosinophil cationic protein were quantified. Cellular expression of κ and λ FLC was characterized in the nasal mucosa of allergic and nonatopic idiopathic rhinitis and control subjects by using immunohistochemistry. Immunopositive cells were phenotyped by using laser microdissection and PCR.

Results: FLC was significantly increased in nasal secretions of subjects with allergy and NARES, and in serum of patients with NARES. Nonatopic patients with allergy showed significantly increased nasal mast cell tryptase and eosinophil cationic protein. FLC-positive cells were significantly increased in allergic and nonatopic mucosa, and were shown to be mast cells and plasma cells.

Conclusion: Nasal FLC is significantly increased in allergic and nonatopic rhinitis nasal mucosa, suggesting a role in nasal hypersensitivity. Further studies are needed to identify which allergens trigger FLC-mediated responses in nonatopic rhinitis.

Clinical Implications

Novel treatment strategies involving blocking FLC might be effective in the treatment of allergic and nonallergic rhinitis.

Introduction

Allergy commonly manifests in the upper and lower airways as allergic rhinitis and asthma, respectively. Allergic rhinitis[1] and asthma[2] appear to share a unified disease pathway[3] attributed to an inflammatory cell cascade involving TH2[4] cells with the production of allergen-specific IgE antibodies from activated B cells (plasma cells). Subsequently, IgE is taken up by receptors on mast cells and when cross-linked by allergen, degranulation occurs with the release of inflammatory mediators[5]. Mast cells are abundant in the nasal mucosa of allergic rhinitis mucosa and submucosal lung tissue,[6] consistent with their role in maintaining inflammation in allergic airway disease. More recently, it has been shown that some patients with nonallergic rhinitis also show increased numbers of mast cells in their nasal mucosa, supporting the proposal that a proportion of nonatopic patients have an inflammatory-type pathology, localized to the nasal mucosa[7,8].

A significant amount of IgE is produced locally in the submucosal region of allergic lung and nasal tissue[9,10] and may account for allergen-specific IgE in subjects with an absence of systemic IgE[11,12]. By definition, nonallergic rhinitis and nonallergic asthma have a pathophysiology that does not involve IgE[13], but reports showing the presence of mucosal inflammatory cells[7, 14-16] and physiological responses[17] compatible with a local allergic-type immune response in some nonatopic subjects confound this definition. Other explanations have been sought for the immediate allergic-type responses found in some patients who apparently have nonatopic rhinitic and asthma in response to allergen challenge. Recently, evidence was provided of a novel and alternative IgE-independent antigen-specific hypersensitivity pathway, mediated by immunoglobulin free light chain (FLC)[18]. Just as passive IgE-mediated hypersensitivity can be transferred to nonatopic individuals, a mouse model has demonstrated that FLC-mediated sensitivity can be conferred to nonsensitized mice. Moreover, it is proposed that the FLC pathway might have a role in asthma because antigen-specific induced mast cell activation and bronchoconstriction has been shown to be associated with functional FLC activity in a mouse model[19]. Although it was shown that FLCs bind to cultured murine mast cells, no direct evidence of FLC binding to mast cells in human tissue has yet been provided.

In healthy individuals, polyclonal (kappa and lambda) FLC are produced in excess, but their precise function remains unknown. In disease states such as myeloma, increased levels of monoclonal FLC are detectable as a consequence of clonal expansion of plasma cells, and furthermore, increased concentration of oligoclonal/polyclonal FLC is seen in a number of autoimmune/inflammatory diseases[22-24]. Because FLCs are involved in mediating skin and airway hypersensitivity[18,19] in mice, we hypothesized that they might serve as candidate

mediators for the allergic responses seen in some systemically IgE-negative subjects with rhinitis, warranting further investigation.

In this study, levels of secreted FLC in nasal secretions were measured. Furthermore, we investigated the presence and localization of FLC-expressing cells in the nasal mucosa of patients with allergic and a form of nonallergic (nonallergic rhinitis with eosinophilia [NARES]) persistent rhinitis, and sought to identify the cell types binding/producing FLC. Our *in vivo* studies showed increased levels of bound and secreted FLC in the nasal mucosa of individuals with persistent rhinitis, irrespective of their atopic status, compared with healthy controls. These findings suggest a role for FLC in chronic upper airway inflammation.

Table 1. Patient profiles for the allergic and nonallergic (NARES) rhinitis groups and normal control group

Group	Gender profile	Age range (Mean)	Mean nasal symptom score (range 0-3)		Serum specific IgE
Normal controls (n = 90)	36M 54F	11-79 (42.6)	Obstruction Secretion Sneezing Itching	1.19 (SD 1.1) 1.16 (SD 0.9) 1.17 (SD 1.0) 0.84 (SD 0.9)	None
Allergic Rhinitis (n = 90)	57M 33F	10-68 (27.1)	Obstruction Secretion Sneezing Itching	2.18 (SD 0.9) 1.74 (SD 0.9) 1.70 (SD 1.1) 1.29 (SD 1.0)	HDM only: 14/90 HDM & S: 24/90 HDM & P: 13/90 HDM & S/P 39/90
NARES (n = 90)	63M 27F	8-92 (36.2)	Obstruction Secretion Sneezing Itching	1.95 (SD 1.2) 1.52 (SD 1.0) 1.20 (SD 1.1) 0.89 (SD 1.0)	None

F, Female; *M,* male
Sensitivity to allergens was assessed by detection of serum specific IgE and patients were categorized into groups showing sensitivity to house dust mite (HDM), seasonal (*S*: timothy grass pollen, birch pollen, herbs), perennial (*P*: storage mites, pet danders, molds).

Materials and Methods

Patient selection

Groups of volunteers were recruited to assess the presence of soluble immunoglobulin FLC, mast cell tryptase (MCT), eosinophil cationic protein (ECP) and IgE in nasal secretions.

Patients were recruited in the Department of Otorhinolaryngology, Head and Neck Surgery, Ludwig-Maximilians-University, Munich, Germany. All subjects underwent an examination by a rhinologist, including nasal endoscopy, atopy-screening test, and if needed CT scans of paranasal sinuses. Rhinosinusitis and/or nasal polyposis constituted specific exclusion

criteria for this study, as did local or systemic anti-inflammatory medication. The number of patients with comorbidity for asthma was as follows, based on self-reporting complaints: 14 of 90, nonallergic rhinitis; 4 of 90, NARES; and 2 of 90, controls without rhinitis. None of the patients with idiopathic rhinitis had asthma. Patients with persistent allergic rhinitis (n=90) who had a positive skin prick test (SPT) to house dust mite (HDM) allergen and serum specific IgE (mean *Dermatophagoides pteronyssinus*-specific IgE, 16.5±10.7U/mL) were compared with patients with NARES (n=90), and nonatopic normal controls (n=90). NARES patients were defined as nonatopic with nasal secretion ECP >300ng/mL. Patient profiles for each of the subject groups are shown in **Table 1**.

To represent the diversity of heterogeneity found in nonallergic rhinitis, a separate group of patients with idiopathic rhinitis of unknown etiology were retrospectively identified from tissue archives belonging to patients previously referred to the Division of Otorhinolaryngology, Queen's Medical Centre, Nottingham University Hospitals Trust, Nottingham United Kingdom, for nasal obstruction. Formalin-fixed tissue blocks of inferior nasal turbinate tissue were obtained from patients with idiopathic rhinitis (n=10; 6 men, 4 women; age range 18-56 years) and compared with groups of patients with persistent allergic rhinitis (n=10; 7 men, 3 women; age range 18-50 years) and a normal control group (n=10; 5 men, 5 women, age range 19-48 years). Strict patient selection criteria were applied as previously described[7]. All patients were free of asthma and had been withdrawn from topical corticosteroids and antihistamines for periods of 28 and 7 days, respectively. Patients with allergy had a relevant medical history of allergic rhinitis and a positive SPT to HDM (*D pteronyssinus*) allergen indicated by a weal greater than 3mm in diameter and HDM specific IgE in the serum (UniCap; Freiburg, Germany). In contrast, the idiopathic rhinitis group had negative SPT and serum specific IgE tests but displayed persistent (perennial) rhinitis symptoms.

The Ethics Committee at each institution approved the study, and informed consent was given by patients and volunteers who participated in this study.

Measurement of total IgE, FLC, MCT, ECP

Patients attending the Department of Otorhinolaryngology, Munich, had nasal secretions collected by the absorption method, as previously described and validated in more than 1700 patients[25] (see this article's **Methods** in the Online Repository [page 61-62]).

Atopic condition was determined by measuring total serum IgE and by using the SX1 atopy screening test (Phadiatop®, both UniCAP, Phadia, Freiburg, Germany), according to the supplier's instructions. Atopic individuals underwent further *in vivo* and *in vitro* allergy tests,

including SPT, nasal allergen provocation, and allergen-specific IgE determination in serum (UniCAP, Phadia, Freiburg, Germany).

Nasal secretions of all patients were analyzed for MCT as a marker of mast cell degranulation, ECP as a marker of tissue eosinophil activation, and total IgE (all UniCAP, Phadia, Freiburg, Germany) in accord with the manufacturer's instructions as previously described[25]. FLC concentrations were measured using an ELISA technique specific for λ and κ FLCs as described[18].

Immunohistochemistry for κ and λ FLC expressing cell counts

Blocks of nasal mucosa were stored at -80^{o}C or fixed in formol calcium for immunohistochemistry (see additional information in this article's **Methods** in the Online Repository [page 61-62]). Sections of nasal mucosa were immunostained for κ and λ FLCs by using validated primary mouse mAbs (kindly donated by Dr A. Solomon, Knoxville, Tenn). The anti-free κ (Fκ-C8) and anti-free λ (Fλ-G9) antibodies have previously been shown to bind specifically only to κ and λ Bence Jones protein fragments, respectively[26, 27] (see this article's **Methods** in the Online Repository for additional information concerning validation and immunohistochemistry [page 61-62]). Cell counts of κ- and λ-positive cells were performed by using an eyepiece graticule by counting the number of cells in alternate fields within the epithelium and submucosal regions using an x40 magnification objective lens. The resulting counts were multiplied by a factor to arrive at cell counts per square millimeter. Frozen sections of nasal mucosa were immunostained for κ and λ FLC for comparison with the paraffin-processed tissue.

Sequential immunostaining

Sequential immunostaining was performed to determine the cell localization of λ FLC-stained cells seen in serial paraffin sections of allergic and nonallergic rhinitic mucosa using the labeled streptavidin biotin immunoperoxidase technique. Alternate sections were immunostained with the anti-λ FLC antibody with intervening sections stained for MCT (DakoCytomation, Cambs, UK), CD68 (macrophages; DakoCytomation), CD138 (plasma cells; DakoCytomation), and CD34 (blood vessels**;** NovaCastra, Newcastle, UK).

Laser microdissection and PCR to investigate the identity of λ FLC-positive cells

To investigate the candidacy of cells positively stained with λ FLC, a rapid immunohistochemistry technique was performed on frozen sections from a single subject with nonallergic nasal mucosa. A PALM Microbeam laser microdissector (Zeiss, Bernreid,

Germany) was used to obtain 500 single cells stained for λ FLC. Total RNA was extracted and converted to cDNA for PCR by using exon-spanning PCR primers for MCT (mast cell-specific), CD138 (plasma cell-specific) and 18s ribosomal RNA. PCR products from the MCT and plasma cell DNA samples were sequenced by using an Applied Biosystems (ABI) 3130 instrument (PerkinElmer, Waltham, Mass; see additional information in this article's **Methods** in the Online Repository for additional information [page 61-62]).

Statistics

The FLCs in nasal lavage secretions and serum were compared in a Mann-Whitney *U* test by using an SPSS program (version 15; SPSS Inc, Chicago, Ill). Correlations between FLC and MCT, ECP and IgE in nasal secretions were investigated by using the Spearman test. Numbers of κ and λ FLC-stained cells were compared in the 3 Nottingham patient groups by using a 2-tailed ANOVA. Subsequently, a follow-on Mann Whitney *U* test was performed on pairs of the patient groups. A *P* value equal to or less than 0.05 was considered significant.

Results

IgE measurements

Total IgE levels were measured in serum and nasal secretions. Significantly increased IgE was found in serum from the allergic group compared with healthy controls (P<0.0001), but not in patients with NARES (P=0.7411). Similarly, levels of nasal IgE were significantly increased in the allergic group (P<0.0001) but not in the NARES group (P=0.3213; **Fig 1**, *A*).

FLC, MCT, and ECP measurements

Levels of κ and λ FLC were measured in nasal secretions of patients with perennial allergic rhinitis (PAR) and NARES and in control normal counterparts. K FLC was significantly increased in nasal secretions of subjects with allergy (P=0.019) and NARES (P<0.0001). A similar increase was also seen for λ-FLC in PAR (P=0.0007) and NARES (P<0.0001) samples (**Fig 1,** *B*). Levels of serum κ and λ FLC were significantly increased in patients with NARES (P<0.05) but not in the allergic group.

The inflammatory mediators MCT and ECP were measured in nasal secretions to assess degranulation of mast cells and eosinophils, respectively, as markers of mucosal inflammation. MCT and ECP levels were significantly increased in allergic and nonallergic secretions (P<0.0001; **Fig 1,** *C*).

Significant associations were seen between secreted κ and λ FLC and secreted MCT (κ, *r*=0.2599; λ, *r*=0.2549) and ECP (κ, *r*=0.3535; λ, *r*=0.3706) for the allergic group (P<0.05). In addition, a significant association was found between κ and λ FLC and nasal IgE (κ, *r*=0.2759; λ, *r*=0.3755) in the PAR group (P<0.05). No such associations were detected in NARES samples.

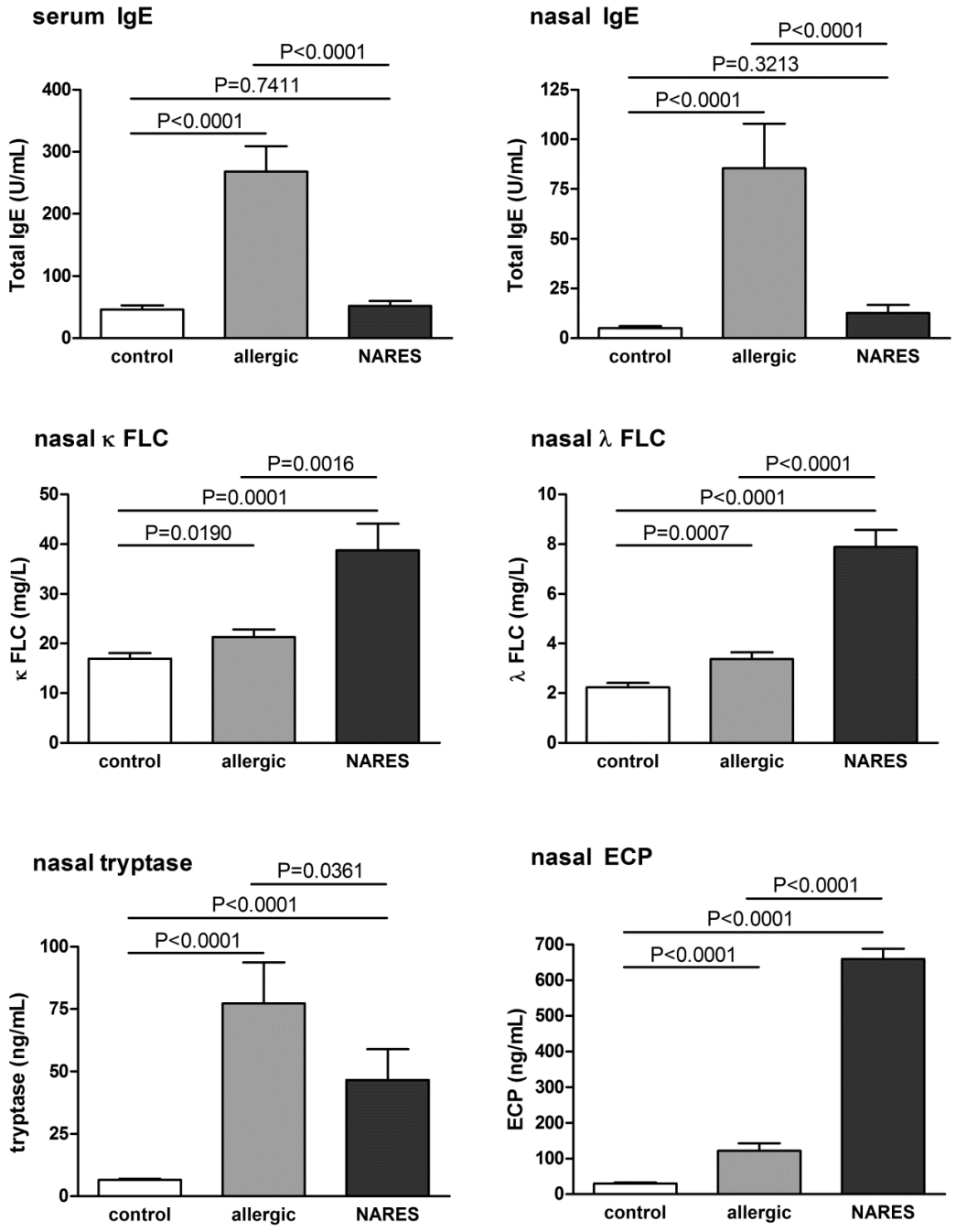

FIG. 1. **A,** Increased serum (P<0.0001) and nasal (P<0.0001) IgE was found in the allergic but not in the NARES or control group. **B,** Levels of nasal kappa and lambda FLC were significantly increased in allergic and NARES patients compared to healthy controls. **C,** The allergic and NARES groups showed increased tryptase and ECP levels compared to control subjects.

Immunohistochemistry for κ and λ FLC

The pattern of κ and λ FLC immunostaining was similar in frozen and paraffin-embedded allergic and nonallergic perennial rhinitis tissues (**Fig 2**) with cells localized to the lamina proprium. More cells stained for λ FLC and the staining was more intense than that obtained with the anti-κ FLC antibody. A significant increase in numbers of κ FLC-stained cells was detected in allergic (P=0.005) and idiopathic rhinitis (P=0.035) mucosa compared with controls. Numbers of λ FLC-stained cells were also increased in allergic (P=0.06) and idiopathic rhinitis (P=0.0001) mucosa (**Fig 3**). In rhinitic mucosa, increased numbers of FLC-stained cells were localized in stroma between the submucosal glands where plasma cells predominate. In the more superficial lamina proprium, FLC-staining was seen in granulated cells with a morphology resembling mast cells or CD68-positive macrophages. Serial sections immunostained with a panel of antibodies indicated localization of FLC to mucosal areas containing plasma cells and mast cells. In addition, a band of FLC staining was seen associated with the epithelial basement membrane and the adjacent basal epithelium, particularly in frozen sections, but the staining did not appear localized to single cells. Some CD34-positive capillaries and small vessels also showed localization of FLC.

Laser microdissection and PCR

Frozen sections of allergic rhinitis mucosa were immunostained for λ FLC to facilitate single cell laser microdissection (see this article's **Fig E1** in the Online Repository [page 63]). Extracted RNA subjected to PCR for MCT, CD138 (plasma cells) and 18s ribosomal RNA resulted in products of expected size (see this article's **Fig E2** in the Online Repository [page 63]). Subsequent sequencing of the putative MCT and CD138 (plasma cell) amplicons confirmed fidelity.

Discussion

In this study, we showed that patients with allergic and nonallergic rhinitis differ significantly from normal healthy controls in having increased numbers of FLC-expressing cells in their nasal mucosa, accompanied by increased levels of secreted FLC. Furthermore, we found FLC is associated with mast cells and plasma cells, supportive of a possible local immune hypersensitivity function.

Allergic rhinitis is defined by IgE-mediated mast cell degranulation, contributing to the hallmark inflammatory symptoms seen in rhinitis. More recently, compelling evidence has shown the importance of locally produced IgE within the nasal mucosa of atopic subjects[28-31]. Interestingly, some apparently nonatopic subjects with rhinitis share similar histologic

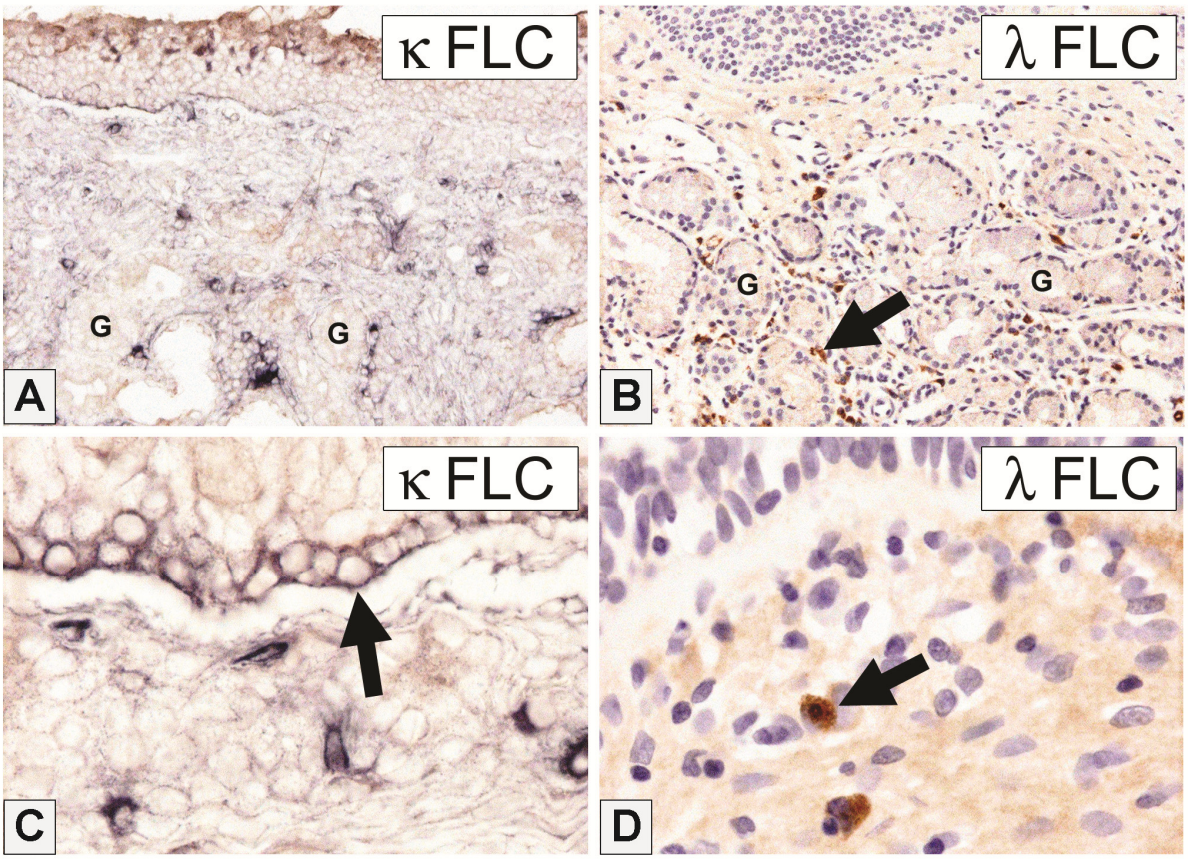

FIG. 2. IHC showed FLC localized to stromal cells adjacent to submucosal glands (*G*) in (**A**) allergic and (**B**) idiopathic nasal mucosa, compatible with localization to plasma cells (arrow). FLC staining was seen in the (**C**) epithelium (arrow) and in (**D**) granulated cells with morphological features of mast cells (arrow) in the superficial lamina proprium.

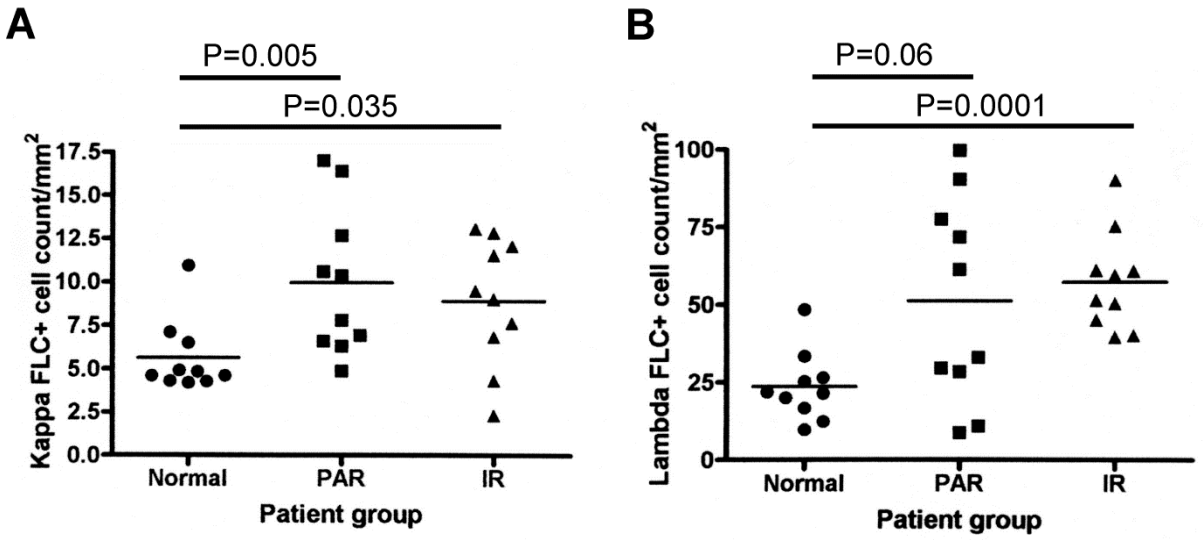

FIG. 3. Increased numbers of (**A**) κ- and (**B**) λ-FLC stained cells appeared in the lamina proprium of nasal mucosa of allergic rhinitis (PAR) and idiopathic rhinitis (IR) mucosa compared to normal control subjects.

mucosal features characterized by increased numbers of mast cells and eosinophils[7]. Included in this group are patients with NARES[32] and idiopathic rhinitis; both are characterized by a pathophysiology involving mast cells and eosinophils[15, 33]. Previously, we showed that some idiopathic subjects have the ability to bind specific allergen to mast cells in their nasal mucosa despite having negative systemic atopic tests for the same allergen. Subsequently, we introduced the term *entopy* to define the presence of a localized immune response in the nasal mucosa of nonatopic rhinitis patients who tested negative with SPTs and RAST[14]. The precise immune pathway responsible for this observation is not known but our findings are consistent with studies that have shown anomalous IgE expression in nonatopic patient nasal secretions. Specific IgE has been identified in nasal secretions from RAST-negative patients with nonallergic rhinitis[11,12,34,35], and symptoms accompanying physiological airway changes have been documented in similar patient groups[17].

An alternative explanation for the immune type responses seen in some subjects with nonatopic rhinitis might be found in non-IgE mediated pathways, possibly involving IgG, because it was previously reported that in chronic sinusitis, increased levels of antifungal IgG antibodies are detectable, resulting in an inflammatory response[36]. More recently, attention has focused on the role of FLC in chronic inflammatory disease. Using a murine model, we previously showed that FLCs appear to function in a manner that is analogous to IgE and have a pivotal role in T cell-mediated immediate and delayed hypersensitivity immune responses[37]. Moreover, there is a suggestion that local FLC production may account for the pathology seen in contact hypersensitivity, multiple sclerosis and rheumatoid arthritis because of the formation of germinal center-like structures reported in some chronic inflammatory diseases[38] including rheumatoid arthritis[39]. Importantly, there is increasing evidence linking FLC with the sensitization of mast cells and a proposed role in asthma[18,19]. In the case of some nonatopic forms of rhinitis, mast cells have been proposed to have surrogate T cell functions in promoting immunoglobulin production in B cells[40].

Ours is the first study to investigate the localization of FLC in allergic and nonallergic rhinitic tissue by using immunohistochemical and ELISA techniques with monoclonal anti-FLC antibodies of high specificity and sensitivity[26,27]. From our animal experiments, we know that lymph nodes are certainly producers of FLC in mice[18], and although likely, it is not known with certainty whether this is the case in human beings. It is known that B lymphocytes localized in nasal mucosa and lymph nodes contribute to systemic and local mucosal IgE levels, respectively, and it is conceivable that FLCs detected in the serum and nasal secretions of our patient cohorts also derive from these cellular sources. Moreover, we showed the apparent localization of FLC to mast cells in idiopathic rhinitic mucosa, leading us to suggest that FLC may function in promoting and supplementing the inflammation seen

in atopic and nonatopic rhinitis forms. Our findings of increased levels of secreted FLC in NARES and FLC-positive cells in patients with idiopathic rhinitis, more so than those identified in allergic rhinitis, are in agreement with our previous observation of increased mucosal mast cells in idiopathic rhinitis[7]. These data suggest that mast cell activation and degranulation could be induced by a FLC-mediated mechanism, but although it is tempting to suggest that FLCs are involved in mediating allergen-induced responses, further studies are required to investigate this possible function. In the perennial allergic group, levels of secreted FLC predictably showed significant association with the granulocyte mediators MCT and ECP, supporting a possible role for their involvement in the allergic inflammatory cascade. In this study, the increased FLC production is most likely a reflection of polyclonal stimulation of B cells. Increases in κ FLC correlated well with increases in λ FLC in the patients with allergy and NARES. No unidirectional (monoclonal) increase in κ or λ FLC was observed. We hypothesize that increased FLC seen in rhinitis may a result of increased polyclonal synthesis of antigen-specific FLC (similar to increased synthesis of specific IgE in patients with allergy) but further studies are needed to test this.

In conclusion, we provide evidence of significantly increased FLC-expressing cells in the mucosa of patients with persistent allergic and idiopathic rhinitis, localized to mast cells and plasma cells. In addition, serum FLC levels are increased in subjects with NARES, and further studies are required to explain the significance of this observation. On this evidence, we propose that FLC may provide an additional non-IgE immune pathway to augment or replace IgE-mediated hypersensitivity in chronic mucosal inflammatory disease. In accord with the recommendations of the EACCI task force[13], the presence of a FLC immune disease pathway in some subjects with idiopathic and NARES rhinitis may require their reclassification as having non-IgE mediated allergic rhinitis. Additional studies are needed to better understand the pathophysiology of nonallergic rhinitis[41]. Among these, further studies are needed to identify which allergens serve as triggers in FLC-mediated mucosal immune responses and could involve measurement of FLC and secretory IgE (sIgE) in nasal secretions from subjects with atopic and nonatopic rhinitis after nasal allergen provocation. If proven, FLC blockade might provide a fresh approach in the treatment of non-IgE-mediated rhinitis, because such antagonists have recently shown promising results in animal models[18, 19, 42, 43].

Acknowledgements

We thank Anne Kane and Claire Boag for assistance in the production of the photographs. Diana Gomes and Sandra Macedo are thanked for their assistance in the determination of FLC in the patient samples. We are grateful to Alan Solomon for providing the FLC-specific antibodies.

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Online Repository

Methods

Measurement of total IgE, FLC, MCT, and ECP

Patients had nasal secretions collected by the absorption method. Briefly, small cone-shaped cotton wool pieces with a length of about 4 cm and a diameter of about 6 mm were placed into the middle meatus of the nose under anterior rhinoscopic view and were left for 10 minutes, followed by centrifugation of the cotton wool strips at 2000*g* (8°C) for 10 minutes.

Immunohistochemistry for κ and λ–expressing cell counts

Blocks of full-thickness nasal mucosa were fixed in 10% formol calcium and processed in paraffin wax. Separate pieces of nasal mucosa from the same block were stored at –80°C. Sections of transversely orientated nasal mucosa were immunohistochemically stained for κ and λ. For immunohistochemistry, 4-μm-thick wax sections were dewaxed and subjected to antigen retrieval by using microwave-heated 0.01 mol/L sodium citrate buffer, pH 6.0. Sections were stained using a labeled streptavidin biotin technique on a TechMate 500 instrument (DakoCytomation, Cambs, United Kingdom [UK]) according to the manufacturer's instructions. The antifree κ (Fκ-C8) and antifree λ (Fλ-G9) antibodies have previously been shown to bind specifically only to κ and λ Bence Jones protein fragments, respectively[1,2]. ELISA assay confirmed an absence of reactivity with total IgG, IgA, and IgM proteins containing the heavy and light immunoglobulin chains[1]. In addition, size exclusion chromatography was used to separate serum proteins, and the FLC antibodies specifically bound only to proteins that represented monomeric and dimeric forms of FLC (22-45 kd).

Frozen sections of nasal mucosa were immunostained for κ and λ for comparison with the paraffin-processed tissue. Briefly, cryosections were fixed in acetone and incubated in a 1:5000 dilution of each FLC antibody for 1 hour. An alkaline phosphatase antialkaline phosphatase technique (DakoCytomation) was used with visualization in nitroblue tetrazolium (Sigma, Poole, Dorset, UK). Negative control sections had the primary antibody either omitted or replaced by an inappropriate mouse mAb of the same immunoglobulin class and concentration.

Laser microdissection and PCR to investigate the identity of λ-positive cells

To investigate the candidacy of cells positively stained with λ, a rapid immunohistochemical technique was performed on frozen sections of a single allergic and a nonallergic nasal

mucosa sample before laser microdissection. Sections were mounted on PALM PEN-membrane slides (PALM Microlaser Technologies, Bernried, Germany), incubated in chilled 70% ethanol for 5 minutes, and then rapidly immunostained by using an Envision peroxidase technique (DakoCytomation) according to the supplier's instructions. RNase-free conditions were maintained by adding 40 U/mL RNase inhibitor (Ambion, Warrington, UK) to the immunochemical reagents and by the use of RNase-free wash buffers.

A PALM Microbeam laser microdissector (Zeiss, Bernreid, Germany) was used to obtain single cells stained for λ in rhinitic nasal mucosa. Approximately 500 single cells were collected in an adhesive collection cap (PALM), and total RNA was extracted by using a Qiagen RNEasy Micro kit (Qiagen, Crawley, UK). cDNA was generated by using a random hexamer primer mix (Promega, Southhampton, UK) and SuperScript III RNase H-reverse transcriptase (Invitrogen, Paisley, Scotland) reaction in accordance with the supplier's recommendations. PCR was performed on the cDNA samples by using exon-spanning PCR primers for MCT (mast cell–specific), CD138 (plasma cell–specific), and 18s ribosomal RNA. PCR reaction mixes (20 µL) contained 1 µL cDNA, 0.5 µL (10 umol/L) forward and reverse primer, respectively, 2.5 µL 10x PCR buffer (Qiagen), 0.5 µL (10 mmol/L) deoxynucleotide triphosphate mix (ABGene, UK), and 0.12 µL Taq polymerase enzyme (Qiagen). The tubes were initially heated to 95°C for 15 minutes, followed by 40 cycles of 94°C for 30 seconds, 56°C for 60 seconds, and then 72°C for 90 seconds before finally being heated to 72°C for 10 minutes in the Techne TC-312 thermocycler (Eurofins MWG Operon, Ebersberg, Germany). The exon-specific primer sequences used were as follows: MCT (forward, cgggagcagcacctctacta; reverse, gagatgttcacgggctcc; amplicons size, 131 bp), CD138 (forward, tgtagtggagctggttgctg; reverse, gtattctcccccgaggtttc; amplicons size, 122 bp), and 18s ribosomal RNA (forward, aaacggctaccacatccaag; reverse, caattacagggcctcgaaag; amplicons size, 112 bp). A negative control omitting cDNA was included. PCR products were assessed by agarose gel electrophoresis and the amplicon bands excised and purified by using a QiaQuick kit (Qiagen); 2 µL of the mast cell tryptase and plasma cell DNA sample was sequenced by using an Applied Biosystems (ABI) 3130 instrument (PerkinElmer, Waltham, Mass) with an ABI BigDye 3 RRX kit according to the manufacturer's instructions.

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Figures

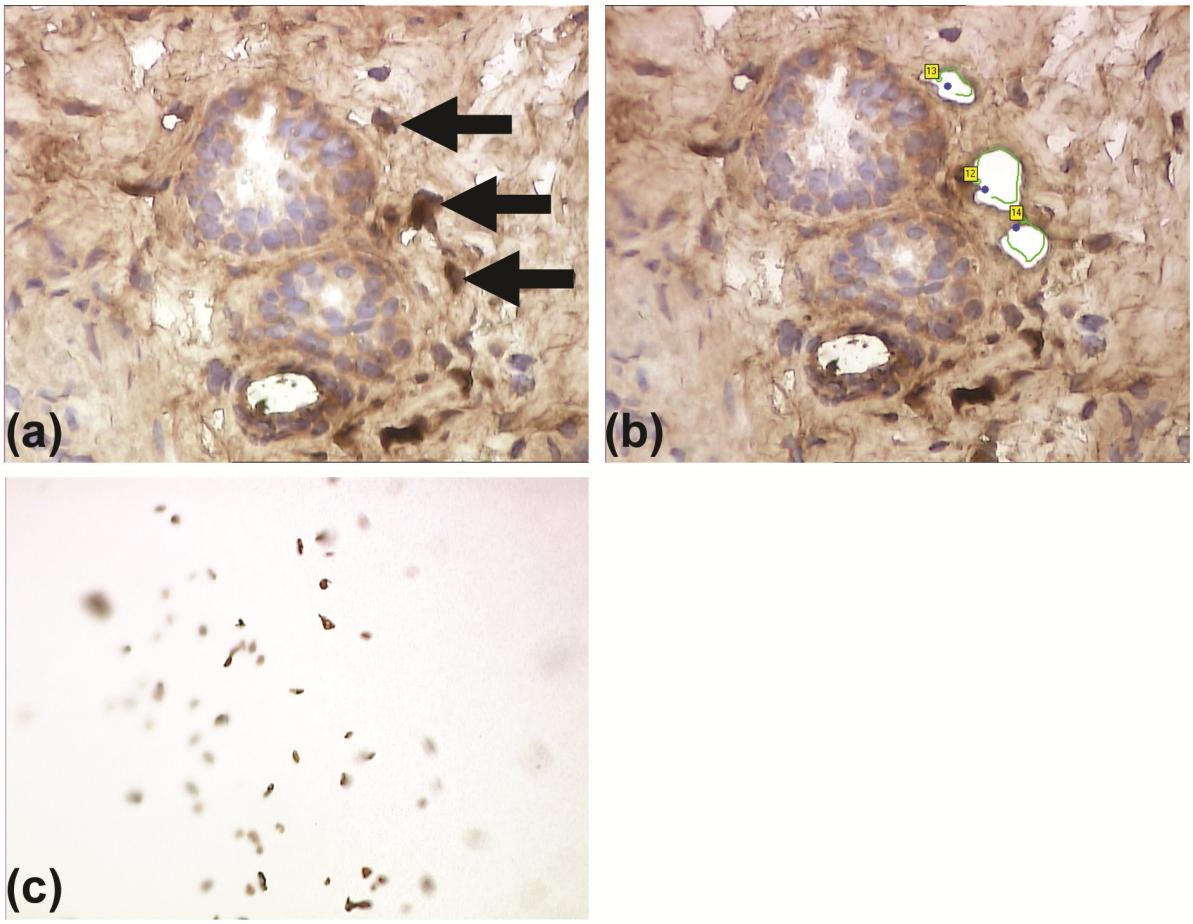

FIG.1. Laser microdissection (LMD) was performed on frozen sections of allergic mucosa after staining for λ-positive cells. **A,** Pre-LMD showing immunostained cells (*arrows*). **B,** Post-LMD. **C,** View of isolated laser-captured cells.

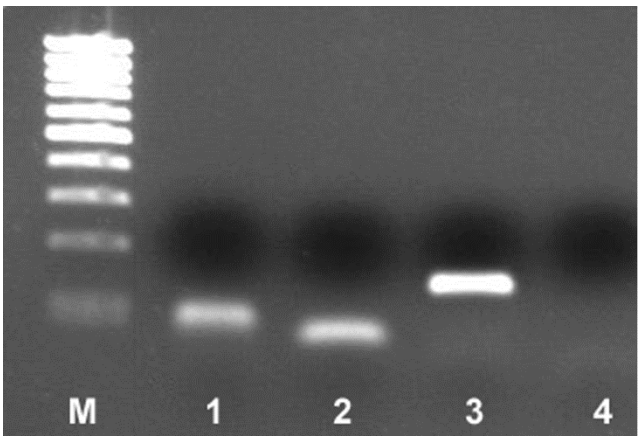

FIG.2. Genotyping of λ-positive cells was performed by using PCR and gel electrophoresis. Primer sequences for the detection of plasma cells (*lane 1*; 122bp), the housekeeping gene 18s rRNA (*lane 2*; 112bp), and mast cell tryptase (*lane 3*;131bp) showed bands of the predicted size compared to DNA (bp) size markers (M). A negative control PCR with cDNA template omitted is shown in *lane 4*.

Letter to the editor:

Are free light chain immunoglobulins related to nasal local allergic rhinitis?

Journal of Allergy and Clinical Immunology, *Volume 126, Issue 3*, *September 2010*, *Page 677*

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The study by Powe et al[1] is the first to demonstrate the localization of free light chains (FLCs) in patients with allergic and nonallergic rhinitis in both tissue and nasal secretions by using immunohistochemical and ELISA techniques and opens up new avenues for the study of the nasal local hypersensitivity response.

Three large groups of subjects are compared: patients with persistent allergic rhinitis (n = 90) who had a positive skin prick test response to house dust mite and serum specific IgE, patients with nonallergic rhinitis with eosinophilia syndrome (NARES; n = 90), and nonatopic healthy control subjects (n = 90). A separate group of patients with idiopathic rhinitis of unknown cause (n = 10) who were retrospectively identified from tissue archives was also included.

The authors provide evidence of “significantly increased FLC-expressing cells in the mucosa of patients with persistent allergic and idiopathic rhinitis, localized in mast cells and plasma cells,” and increased FLC serum levels in subjects with NARES. They propose that “FLC may provide an additional non-IgE immune pathway to augment or replace IgE-mediated hypersensitivity in chronic mucosal inflammatory disease”. Accepting that the mechanisms involved require further study, we nevertheless believe that a few comments are warranted, given the relevance in this context of both idiopathic rhinitis and NARES.

We wonder whether some of the patients with allergic and nonallergic rhinitis included in this study could have had local allergic rhinitis (LAR)? A number of studies have shown the existence of a new form of LAR[2], or entopy[3], with local production of specific IgE and a positive nasal allergen provocation test response in patients previously given diagnoses of idiopathic rhinitis and NARES[2,4]. These patients might present with normal or low levels of total IgE in nasal secretions[4], but we do not believe this is sufficient to discriminate the existence of nasal IgE-mediated hypersensitivity. In the report by Powe et al[1],no study was

made of the response on nasal allergen provocation tests or the determination of specific IgE to aeroallergens in nasal secretions of the allergic and nonallergic patients studied. Might there have been a local production of specific IgE, as we have seen in our studies?

Another question concerns whether FLC levels are associated with nasal mucosal inflammation or a local hypersensitivity response to aeroallergens. It would be interesting to conduct further comparative studies between patients with LAR or entopy and patients with idiopathic rhinitis or NARES without a local hypersensitivity response.

The authors suggest that “mast cell activation and degranulation could be induced by a FLC-mediated mechanism.” We recently detected that patients with LARs who were sensitized to grass pollen have an important and persistent mast cell activation specifically induced by nasal exposure to the aeroallergen[5]. Thus further studies are needed to investigate the possible function of FLCs in local hypersensitivity to aeroallergens.

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Reply

Journal of Allergy and Clinical Immunology, *Volume 126, Issue 3*, *September 2010*, *Pages 677-678*

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We acknowledge the suggestion made by Rondón et al[1] that some of the patients with nonallergic rhinitis investigated in our study might have "entopy"[2] (local mucosal allergy), and for clarity, this will be defined. Entopy is the presence of local mucosal allergy in the absence of systemic markers of atopy, and although it is commonly interpreted that this is IgE mediated, our original report does not implicitly specify this. Accordingly, although patients with allergic rhinitis undoubtedly have a local nasal mucosal IgE production component that might or might not contribute to the expression of atopy, they do not satisfy the definition of entopy because they are atopic.

The emphasis of our present study is to provide a possible mechanism to explain nasal hypersensitivity in nonatopic subjects showing signs of local mast cell activation, as demonstrated by increased tryptase concentrations in nasal lavage specimens. We have shown that in these patients increased local concentrations of free light chains (FLCs) are present, and based on our previous work, we suggest that these FLCs might be involved in antigen-specific mast cell activation[3]. Indeed, Rondón et al[4] have shown that nonatopic patients might present with normal or low levels of total IgE but, importantly, have increased specific IgE levels in nasal secretions, and as the authors state, measurement of total IgE might not be sufficient to discriminate the existence of nasal IgE-mediated hypersensitivity. In our study we have analyzed nasal secretions from patients with nonatopic rhinitis with eosinophilic syndrome for the presence of specific IgE but no specific IgE to seasonal or perennial allergens was found (as stated in Table I of the article by Powe et al[5]). However, when we analyze concentrations of nasal total IgE on an individual basis (**Fig 1**), it is evident that a small percentage of the patients with nonatopic rhinitis with eosinophilic syndrome have increases in local total IgE concentrations (with undetermined specificity), which could point to an IgE-driven hypersensitivity in these subjects.

We agree that further studies are needed to investigate the possible function of FLCs in local hypersensitivity to aeroallergens. A good starting point would be to more closely define the nonallergic rhinitis cohort by performing nasal allergen provocation tests with common allergens to positively select a more homogenous responsive study group. Analysis of nasal fluids for FLCs and IgE after nasal allergen provocation tests would categorize subjects into (1) IgE producers, (2) IgE and FLC producers, (3) FLC producers, and (4) mediator-negative

subjects. By using our definition, groups 1 to 3 are entopic, whereas group 4 (if they exist) has idiopathic rhinitis.

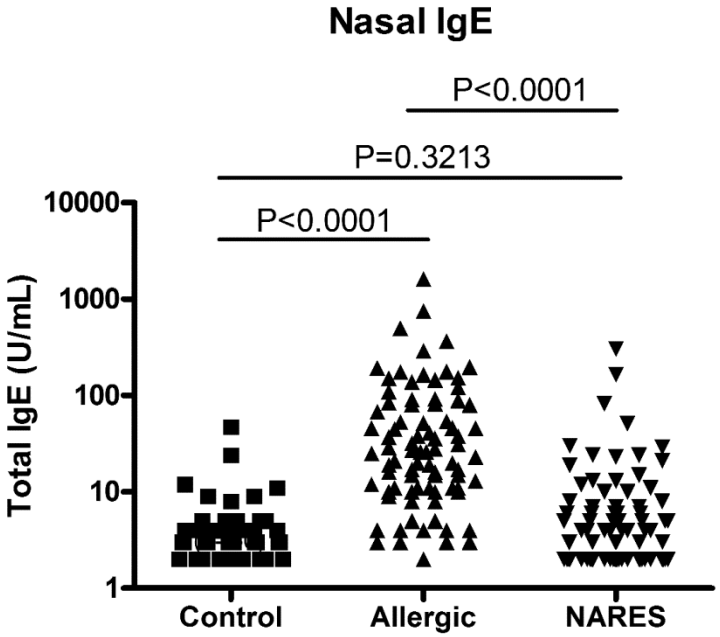

Fig 1. A small proportion of nonatopic patients with nonatopic rhinitis with eosinophilic syndrome (NARES) show increased local mucosal total IgE levels (with undetermined specificity) in nasal secretions, which could point to an IgE-driven hypersensitivity in these subjects.

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4

Expression of immunoglobulin free light chains is locally increased in chronic rhinosinusitis with nasal polyps and can be downregulated by anti-IL-5 treatment

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Abstract

Background: Free light chain (FLC) concentrations are demonstrated to be increased in different inflammatory disorders and are proposed to mediate mast cell-dependent immune responses. A role for mast cells is suggested in chronic rhinosinusitis with nasal polyposis (CRSwNP), which is characterized by a local Th2-inflammatory response. However, clear mast cell activating factors are not always apparent.

Objective: In this study the presence of FLCs in CRSwNP and CRSsNP patients was investigated and the effect of different treatments on FLC expression was analyzed.

Methods: Nasal tissue, nasal secretions and serum of control patients, patients with CRSwNP and CRSsNP (CRS without nasal polyps) were analyzed for presence of kappa and lambda FLC. The expression of FLCs in nasal polyp tissue was investigated by using immunohistochemistry. In addition, FLC was measured in serum and nasal secretions of nasal polyp patients treated with methylprednisolone, doxycycline, mepolizumab or placebo.

Results: FLC concentrations were increased in nasal secretions and mucosal tissue homogenates in patients with chronic rhinosinusitis and this effect was most prominent in CRSwNP patients. Immunohistochemical analysis confirmed the increased FLC concentrations in nasal polyp tissue. In CRSwNP patients, treatment with methylprednisolone or anti-IL5 resulted in reduction of systemic and local FLC concentrations, respectively.

Conclusion: The presence of FLC in CRSwNP and CRSsNP suggests a possible role in mediating the local immune reaction in the paranasal cavities. Furthermore, the decrease of local FLCs after treatment with anti–IL5 presumes that IL-5 creates an environment that favors FLC production.

Clinical implications: Attenuation of FLC production nasal tissue of CRS patients, especially those patients having nasal polyposis, might lead to reduced inflammation and nasal polyposis.

Introduction

Chronic rhinosinusitis (CRS) is a chronic inflammatory disorder of the nose and paranasal cavities, with an estimated prevalence of 10.8% in Europe.[1] The diagnosis of CRS is based on patient history, clinical symptoms and completed by nasal endoscopy and CT scan imaging of the sinuses.[2] Currently, this disease is divided in two different entities based on histology and inflammatory patterns: CRS with (CRSwNP) and without nasal polyps (CRSsNP). Nevertheless, mechanisms underlying etiology are still poorly understood.

CRSsNP is considered a fibrotic disease characterized by a Th1 cell mediated inflammation with high numbers of neutrophils, but low percentages of eosinophils, macrophages, and basophils.[3, 4] In addition, high concentrations of transforming growth factor-β1 (TGF-β1) and interferon-γ (IFN-γ) are found.[3, 5] Nasal polyps observed in CRSwNP are benign oedematous masses protruding from the nasal and paranasal mucosa. Most Caucasian CRSwNP patients have a massive polyp infiltration of inflammatory cells, mainly eosinophils [6]. The local Th2 polarization seen in these polyps is characterized by high levels of eosinophilic markers (eosinophil cationic protein (ECP) and eotaxin) and IL-5, and local production of polyclonal IgE.[4, 5, 7, 8]

Mast cells are primarily known for their important role in acute IgE-mediated allergic reactions as seen in anaphylaxis, asthma and rhinitis. Mast cell activation can result in immediate release of granule-stored mediators, and a large array of cytokines, chemokines, and growth factors several hours after activation.[9] The involvement of IgE in nasal mast cell activation in CRSwNP patients is supported by the local polyclonal IgE production as indicated by their presence in the stroma and the epithelium of nasal polyps independent of atopy.[10-13] Besides, its functional role is suggested in *ex vivo* experiments showing nasal polyp mast cell activation upon IgE-crosslinking using anti-IgE, which could be prevented using Syk inhibitors. The observed resulting increase in mediator release in polyp tissues was significantly higher compared to control inferior turbinate tissue stimulation.[14-16] The continuous release of mast cell mediators has shown to enhance the recruitment of eosinophils in nasal polyp tissue.[17] In contrast to CRSwNP, mast cell numbers in nasal mucosa tissue from CRSsNP patients are not significantly different from healthy control tissue and putative functional differences are unknown.[18]

Next to IgE, a different mechanism of antigen-specific mast cell activation is described mediated by immunoglobulin free light chains (FLCs).[19] B cells produce light chains (either of kappa (κ) or lambda (λ) isotype) that can either be incorporated into immunoglobulins or secreted as unbound FLCs. As a consequence, FLCs can be found in many biological fluids like serum, urine, nasal secretions, and synovial fluid and concentrations are increased in

many inflammatory disorders, including asthma and rhinitis.[20-23] Interestingly, high FLC concentrations are shown to correlate with an increased disease severity in rheumatoid arthritis and multiple sclerosis.[22, 24, 25]

In the present study we investigate the presence of FLCs nasal secretions and tissue of CRS patients, especially in those suffering from nasal polyposis. Furthermore, the treatment effect of different treatment options on FLC was explored.

Table 1. Baseline demographic and clinical characteristics of control subjects, and CRSsNP and CRSwNP patients.

	Control (n=25)	**CRSsNP (n=46)**	**CRSwNP (n=41)**	**p-value**
Age (yr), mean (SEM)	29.92 (2.44)	42.96 (2.08)	45.59 (1.82)	**0.030**[a]
Men/women, n/n	11/14	26/20	26/15	0.317[b]
Length (cm), mean (SEM)	170.00 (1.71)	172.25 (1.56)	173.52 (1.64)	0.529[a]
Weight (kg), mean (SEM)	66.39 (2.13)	72.38 (2.07)	77.00 (2.41)	0.074[a]
Allergy, n (%)	7 (28)	18 (39)	19 (46)	0.338[b]
Asthma, n (%)	4 (16)	8 (17)	16 (39)	**0.046**[b]
Aspirin intolerance n (%)	0 (0)	1 (2)	3 (7)	0.496[b]

Differences between groups were analyzed for each parameter and corresponding p-values are indicated ([a]oneway ANOVA test , [b]exact χ^2 –test)

Methods

Patients

The analysis of FLC concentrations in tissue homogenates, nasal secretion and serum was performed on 112 subjects, included at the Department of Otorhinolaryngology of the University Hospital Ghent, Belgium, between May 2007 and December 2009. 25 control subjects, 46 patients with CRSsNP and 41 patients with CRSwNP participated. The diagnosis of CRS was based on the European position paper on rhinosinusitis and nasal polyposis.[2] To differentiate between CRSwNP and CRSsNP, endoscopic evidence of polyps is necessary. Both groups of patients were allowed to participate if they had concomitant allergy or asthma. Control patients were non allergic subjects who underwent routine septal surgery for nasal obstruction. Patients with parasitic infection, cystic fibrosis, primary ciliary's dysfunction, Kartagener's syndrome were excluded. If patients received oral steroids or antibiotics during the month before inclusion, patients could not participate. All patients appeared in good health.

Treatment effect on serum and nasal secretion FLC concentrations was analysed in patients included in two randomized controlled trials investigating three alternative treatment options for CRSwNP as described previously.[26, 27]. The reduction in total nasal endoscopic polyp score (TPS) was the primary endpoint of these studies. Polyps were evaluated on each side by nasal endoscopy and graded based on polyp size. The total nasal polyp score is the sum of the right and left nostril scores.[27] In brief, the following groups were studied:

In the first study, 14 patients received methylprednisolone (32 mg/d on days 1-5; 16 mg/d on days 6-10; 8 mg/d on days 11-20), 14 received doxycycline (200mg on day 1; 100 mg/d on days 2-20) and 19 received placebo (lactose for 20 days) in a double-blind placebo controlled setting. Nasal secretion and serum was analyzed at baseline and two weeks after treatment.[27]

The second study included 20 patients, who received mepolizumab (anti-IL-5; 2 single IV injections (4 weeks interval) of 750mg)), and 10 patients, who received placebo) in a double-blind placebo controlled setting. The primary endpoint was 8 weeks after the first administration of medication.[26]. The ethical committee of the Ghent University Hospital, Ghent, Belgium approved the studies, and written informed consents were obtained from all subjects.

Collection of serum, nasal secretions, and tissue homogenates

Blood was collected by performing a standard venipuncture (Serum gel separator tubes Terumo Venosafe, Ref VF-106SAS). Blood was centrifuged for 15 minutes at 1500 g at 4°C after allowing it to clot for 15-30 minutes. Serum was stored in aliquots at -20°C until further analysis. Nasal tissue (nasal polyps or inferior turbinates) were collected during routine sinus (FESS) and septal surgery, respectively. All samples were immediately processed, separated and stored in aliquots at –80°C until analysis. Prior to analysis, nasal tissue was weighed, 0.9% NaCl solution with a protease inhibitor cocktail (Roche Diagnositics, Vilvoorde, Belgium) was added (1 mL /0.1g tissue), and tissue was homogenized with a mechanical homogenizer (B. Braun Melsungen, Germany) as described previously.[28] After homogenization, the suspensions were centrifuged at 1500 g for 10 minutes at 4°C, and the supernatants separated.

Nasal secretions were collected by placing sinus packs (IVALON 4000 plus 3.5x0.9x1.2cm surgical product M-Pact, Eudora, Kan) in both nasal cavities for 5 minutes as described previously.[29]

Measurement of immunoglobulin free light chains in tissue homogenates, nasal secretions, and serum

Total κ- and λ-FLC concentrations were determined in all supernatants and sera using an ELISA adapted from Abe *et al.*[30, 31] In brief, plates were coated (o/n; 4°C) with goat-anti mouse IgG (M4280, Sigma, Zwijndrecht, the Netherlands), blocked (1 hour; RT), and incubated with mouse-anti human κ- or λ-FLC (obtained from Dr. A. Solomon, Tennessee, US). After incubation with different dilutions of samples and standards (The BindingSite, Birmingham, UK), plates were incubated with HRP-labelled goat $F(ab')_2$-anti human κ- or λ-light chain antibodies (AHI1804 and AHI1904, respectively, Biosource, USA). TMB was used as a substrate. Per sample, at least three data points within the range of the standard curve were used to estimate the FLC concentration.

Different inflammatory parameters were analyzed by ELISA (IL-5, IFN-γ, IL-8, and MPO; R&D Systems, Minneapolis USA) and Uni-CAP system (ECP, total and specific SAE IgE; Pharmacia, Uppsala, Sweden) according to manufacturer's instructions.

Immunohistochemistry

For staining of FLCs, cryostat sections were used. Specimens were snap frozen in liquid nitrogen cooled methyl butane and stored at -80°C. Cryostat sections were prepared (6μm) and mounted on SuperFrost Plus glass slides (Menzel GmbH & Co. KG, Braunschweig, Germany). Other stainings were performed on 5μm thick formalin-fixed, paraffin embedded tissue sections mounted on poly-L-lysin-coated slides. Paraffin sections were subjected to heat induced antigen retrieval (S1700, Dakocytomation, Haverlee, Belgium) for 25 minutes prior to staining. In short, sections were blocked with PBS-T/3%BSA/3% normal goat serum for 1 hour, and incubated for 1 hour with the following primary antibodies diluted in blocking buffer: mouse-anti-human kappa FLC (Fκ-C8) and mouse-anti-human lambda FLC (Fλ-G9) (both obtained from Dr. A. Solomon, Tennessee, USA), mouse-anti-human CD138 (clone MI15, Dakocytomation, Haverlee, Belgium), rabbit-anti-human CD20 (clone BV11, Abcam, Cambridge, UK), and mouse-anti-human tryptase (clone AA1, Dakocytomation, Haverlee, Belgium). 2 different isotype control antibodies were included (mouse IgG1: X0931, Dakocytomation, Haverlee, Belgium; 02-6100, Invitrogen, Breda, the Netherlands). Tissues were subsequently incubated with Alexa Fluor 568 goat-anti-mouse IgG or Alexa Fluor 488 goat-anti-rabbit IgG (Invitrogen, Breda, the Netherlands) for 1 hour. Nuclei were counterstained with diamidino-phenylindole (DAPI, Invitrogen, Breda, the Netherlands). Sections were viewed with an Eclipse TE2000-U inverted microscope (Nikon) and images were analyzed by using NIS elements BR 2.3 (Nikon). Staining intensity of FLC in nasal

polyps and mucosa was analyzed by using Image J software (version 1.44p; National Institute of Health, USA). Number of positive cell counts and staining intensities are mean values based on 5 randomly selected fields within each tissue.

Table 2. Different inflammatory parameters in tissue, nasal secretion and serum in control subjects, CRSsNP and CRSwNP subjects

	Control (n=25)	Control vs CRSsNP	CRSsNP (n=46)	CRSsNP vs CRSwNP	CRSwNP (n=41)	CRSwNP vs Control
Tissue:						
Total IgE (kU/l)	37.64 (26.32)	**<0.0001**	101.63 (36.61)	**<0.0001**	636.72 (185.84)	**<0.0001**
SAE-IgE (kU/l)	0.00 (0.00)	0.224	2.07 (1.50)	**0.027**	4.15 (2.39)	**0.005**
IL-5 (pg/ml)	3.54 (0.54)	**0.013**	35.19 (11.48)	**<0.0001**	240.52 (59.00)	**<0.0001**
ECP (µg/l)	246.86 (83.69)	**<0.0001**	3010.35 (573.08)	**<0.0001**	9374.16 (1617.03)	**<0.0001**
IL-8 (pg/ml)	1488.01 (346.30)	**0.008**	3400.52 (727.29)	0.823	4991.62 (1423.05)	**0.003**
IFNγ (pg/ml)	52.70 (5.51)	0.074	150.11 (45.77)	0.549	152.81 (52.56)	0.245
MPO (ng/ml)	1630.23 (336.06)	**0.028**	4781.86 (1445.93)	0.539	3517.75 (660.83)	**0.005**
Nasal secretion						
Total IgE (kU/l)	11.63 (4.89)	0.712	16.50 (5.57)	**0.021**	191.11 (109.02)	**0.042**
ECP (µg/l)	234.89 (112.06)	0.252	256.80 (42.34)	0.172	441.50 (98.81)	**0.032**
MPO (ng/ml)	2872.38 (750.09)	0.72	6039.32 (1094.44)	0.578	6482.15 (1179.03)	**0.029**
Serum						
Total IgE (kU/l)	183.78 (88.70)	0.472	257.63 (124.91)	0.186	231.58 (69.12)	0.101
SAE-IgE (kU/l)	0.44 (0.31)	0.111	0.397 (2.02)	0.245	0.97 (0.24)	0.258
ECP (µg/l)	13.34 (2.25)	**0.032**	22.21 (3.17)	0.200	25.31 (2.86)	**0.002**

Differences between groups were analyzed for each parameter by Mann-Whitney U test and corresponding p-values are indicated. All numbers represent mean (SEM).

Statistical analysis

Baseline FLC concentrations in mucosal tissue homogenates, nasal secretions and serum were compared using either the Mann-Whitney test for comparing 2 groups, or the Kruskal-Wallis test for comparison of 3 groups. The latter analysis was followed by a post-hoc Dunn's multiple comparison test on all patient groups. Effects of treatment on FLC concentrations were analyzed using the Wilcoxon matched-pairs signed rank test (nasal secretions and tissue homogenates), or a paired t-test (serum samples). Correlations

between all parameters were determined by the Pearson or Spearman correlation coefficient (the former only in case serum FLC concentrations were correlated). P-values <0.05 were considered statistically significant. All analyses were performed using GraphPad Prism, version 5.03.

Results

Demographic and clinical features

112 subjects were included for measurement of FLC and different inflammatory parameters in tissue homogenates, nasal secretions and serum. Baseline demographic and clinical characteristics are comparable between all groups except for age and asthma (Table 1). Since FLC concentrations are hardly influenced by age in adults,[32-34] this observed difference in age is not likely to bias current results. Patients with CRSwNP have a higher risk for developing asthma, which is demonstrated by more asthmatics in the CRSwNP group compared to CRSsNP and controls. Concentrations of the analyzed inflammatory parameters in the different disease groups are shown in table 2.

No significant differences in baseline characteristics between different treatment groups were observed [26, 27].

FLC concentrations are increased locally in mucosal tissue and nasal secretions in patients with CRS

κ-FLC concentrations in mucosal tissue homogenates in CRSsNP subjects (mean±SEM; 98.4±15.1 µg/mL) and CRSwNP subjects (198.0±37.0 µg/mL) were highly increased compared to control subjects (31.7±5.7 µg/mL; p<0.001 for both). The difference in κ-FLC concentrations between the CRSwNP group and the CRSsNP group was significantly different (p<0.05). Similar differences were found for λ-FLC concentrations between control subjects (37.5±7.2 µg/mL), CRSsNP subjects (132.5±26.8 µg/mL) and CRSwNP subjects (199.2±28.1 µg/mL) (Figure 1A). In nasal secretions, κ-FLC concentrations were comparable in control (8.8±1.8 µg/mL) and CRSsNP (14.5±2.0 µg/mL) subjects, but both were significantly lower compared to concentrations in the CRSwNP group (42.4±9.6 µg/mL; p<0.001 compared to control, p<0.05 compared to CRSsNP). λ-FLC concentrations were comparable in control (6.2±1.4 µg/mL) and CRSsNP (15.0±2.5 µg/mL) subjects, but only controls had significantly lower FLC concentrations compared to the CRSwNP group (22.3±3.4 µg/mL; p<0.01) (Figure 1B). Total serum FLC concentrations were comparable in CRS patients with (κ: 29.1±1.9 mg/L, λ: 24.0±1.8 mg/L) and without (κ: 26.4±1.4 mg/L, λ: 21.3±1.5 ±1.9 mg/L) nasal polyposis and control subjects (κ: 25.9±2.4 mg/L, λ: 20.7±1.9 mg/L) (Figure 1C).

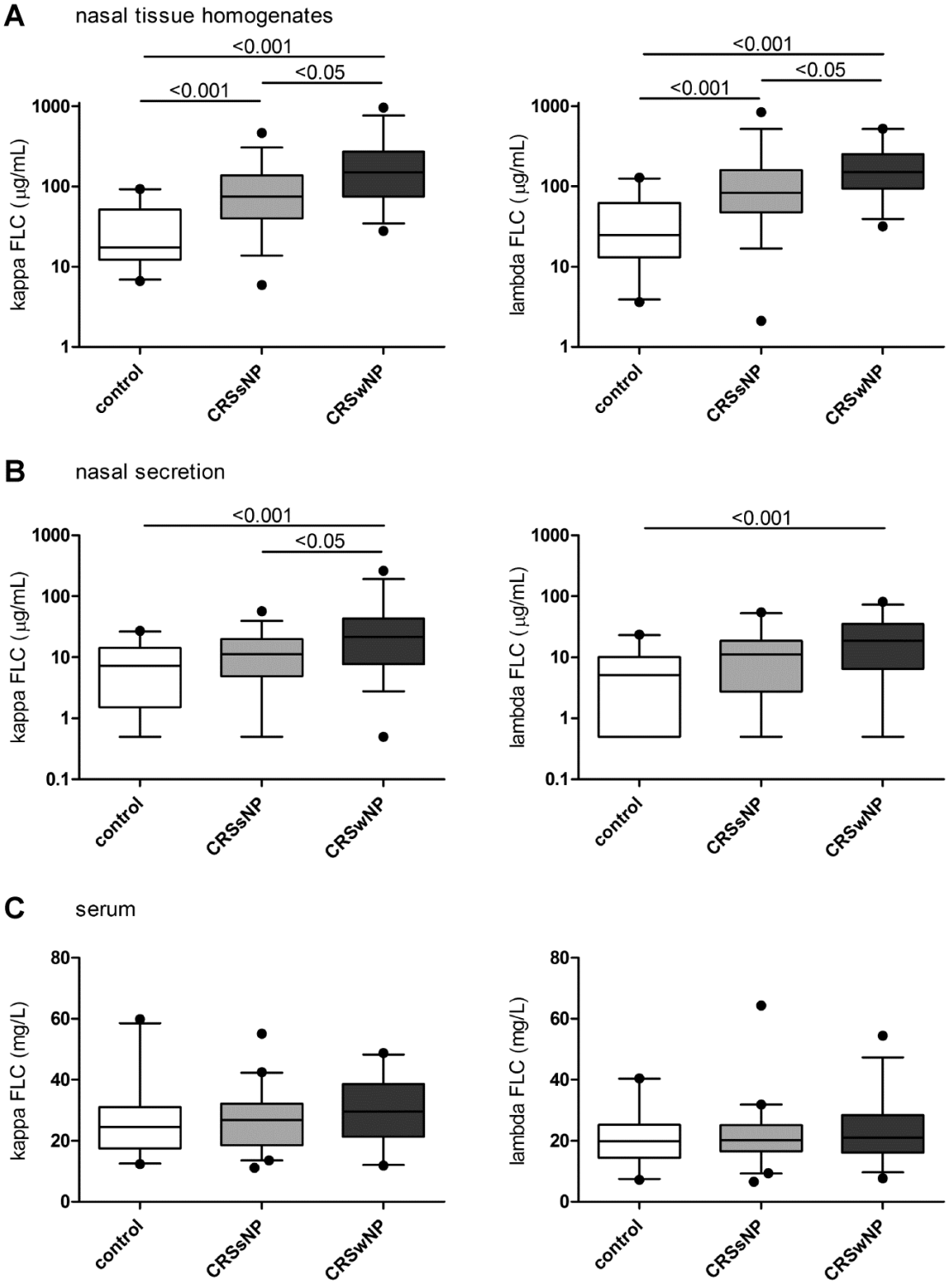

Figure 1. Comparison of FLC concentrations between control subjects, chronic rhinosinusitis patients without (CRSsNP) and with nasal polyps (CRSwNP) in nasal tissue homogenates (**A**), nasal secretion (**B**), and serum (**C**). FLC concentrations are significantly increased at local sites of inflammation in CRSsNP, and even more apparent in CRSwNP (**A** and **B**). *Box-and-whisker plot*s represent median and 5-95 percentiles. Outliers are displayed as separate points.

Correlations between κ-FLC and λ-FLC concentrations measured in nasal secretions (control: $p<0.0001$, $r=0.94$; CRSsNP: $p<0.0001$, $r=0.71$; CRSwNP: $p<0.0001$, $r=0.81$),

mucosal tissue homogenates (control: p<0.0001, r=0.90; CRSsNP: p<0.0001, r=0.72; CRSwNP: p<0.0001, r=0.76), and serum (control: p=0.0003, r=0.90; CRSsNP: p<0.0001, r=0.82; CRSwNP: p<0.0001, r=0.86), were highly significant in all groups.

Analysis of correlations between FLC and different inflammatory parameters in tissue indicated a significant correlation between FLC and total IgE (κ: p= 0.002; λ: p= 0.003) and IL-5 (λ: P=0.004) in CRSwNP. In CRSsNP, a significant correlation between MPO and FLC was found (κ: p= 0.002). In contrast, no significant correlations were found in control patients.

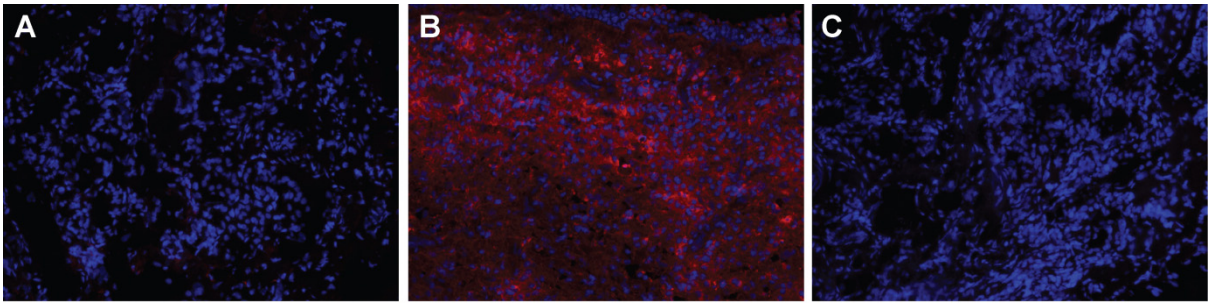

Figure 2. The presence of κ- and λ-FLC (double staining) in nasal polyp tissue and control nasal mucosa by means of immunohistochemistry. Control nasal mucosa showed little FLC positive cells (**A**), whereas nasal polyp tissue showed highly diffuse and enhanced FLC staining (**B**). Isotype control antibodies did not show any positive staining in polyp tissue (**C**).

FLCs are diffusely distributed in nasal polyp tissue

Since FLCs were highly present in nasal polyp tissue, the localization of κ- and λ-FLCs within nasal polyp tissue and nasal mucosa from control subjects was analyzed using immunohistochemistry. Control nasal mucosa showed little FLC positive cells (Figure 2A), whereas nasal polyp tissue showed highly diffuse and enhanced FLC staining (Figure 2B). Isotype control antibodies did not show any positive staining in polyp tissue (Figure 2C). Some bright FLC-positive cells could be detected in both control and polyp tissue, but quantification of the number of these cells showed no significant differences (Figure 3A). These cells did not show double staining with tryptase. Practical limitations hampered double staining for FLC and CD138. FLC staining in polyp tissue is largely diffuse, probably resulting in an underestimation of the number of FLC-positive cells. Numbers of tryptase-positive mast cells, B cells, and plasma cells were all significantly higher in polyp tissue as compared to control tissue (mast cells: p=0.05; B cells: p=0.02; plasma cells: p=0.008, data not shown).

Analysis of FLC staining intensities showed a significant difference for λ-FLC between both groups (κ-FLCs: control: 11.0±2.8; NP: 21.2±3.3; p=0.1, λ-FLC: control: 10.1±1.5; NP: 20.1±2.9; p=0.03; Figure 3A). Correlations between the number of FLC-positive cells and

staining intensity were highly significant for both κ-FLCs (p=0.0027; r=0.67, Figure 3B) and λ-FLCs (p=0.0024; r=0.67, Figure 3B). Both κ-and λ-FLC staining intensities highly correlated with FLC concentrations in NP tissue homogenates from the same tissue analyzed with the FLC ELISA (κ-FLCs: p<0.001; r=0.87, λ-FLC: p=0.001; r=0.68, Figure 3C). A significant correlation with the number of FLC-positive cells was only observed for κ-FLCs (p=0.003; r=0.80).

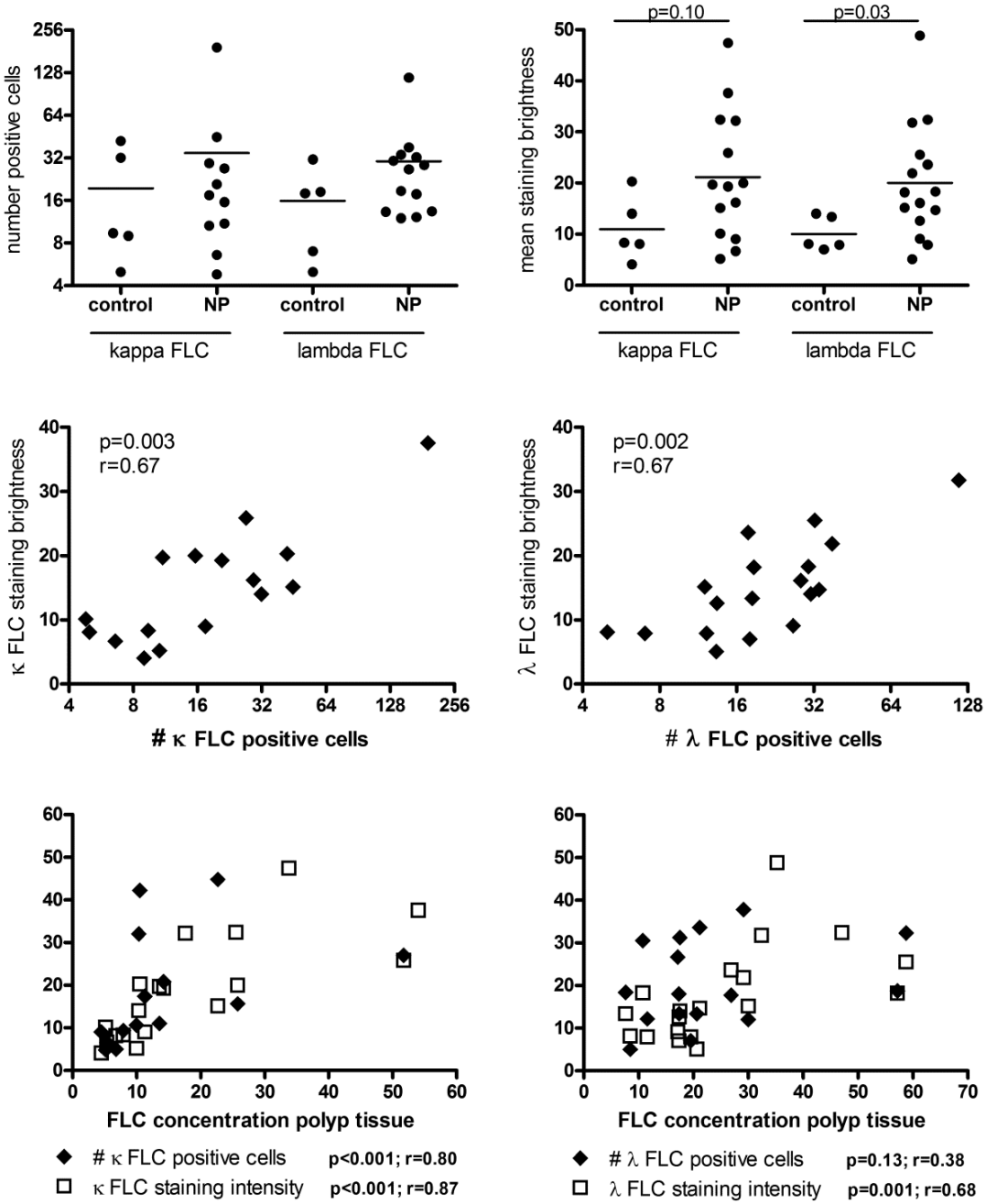

Figure 3. Immunohistochemical analysis of nasal polyp tissue and control nasal mucosa did not show a difference in FLC-positive cell numbers, whereas FLC staining intensity was higher in NP tissue (**A**). Staining brightness significantly correlated with the number of FLC-positive cells (B). Moreover, both parameters correlated with FLC concentrations as measured by means of ELISA in tissue homogenates derived from the same tissue (**C**).

Serum and nasal FLC concentrations after treatment with methylprednisolone and doxycycline in CRSwNP

Serum FLC concentrations were significantly decreased after treatment with the systemic corticosteroid methylprednisolone, a general treatment option for nasal polyposis (κ-FLCs: before: 35.4±2.76; after: 28.6±2.62, p<0.001; λ-FLC: before: 33.8±2.43; after: 29.6±2.27, p=0.0048). Placebo treatment did not affect FLC serum concentrations (κ-FLCs: before: 34.5±3.31; after: 36.3±2.78, p=0.59; λ-FLC: before: 30.3±3.25; after: 32.6±2.95, p=0.19) (Figure 4A-B). FLC concentrations in nasal secretions were unaffected. Doxycycline treatment did not affect serum or nasal FLC concentrations.

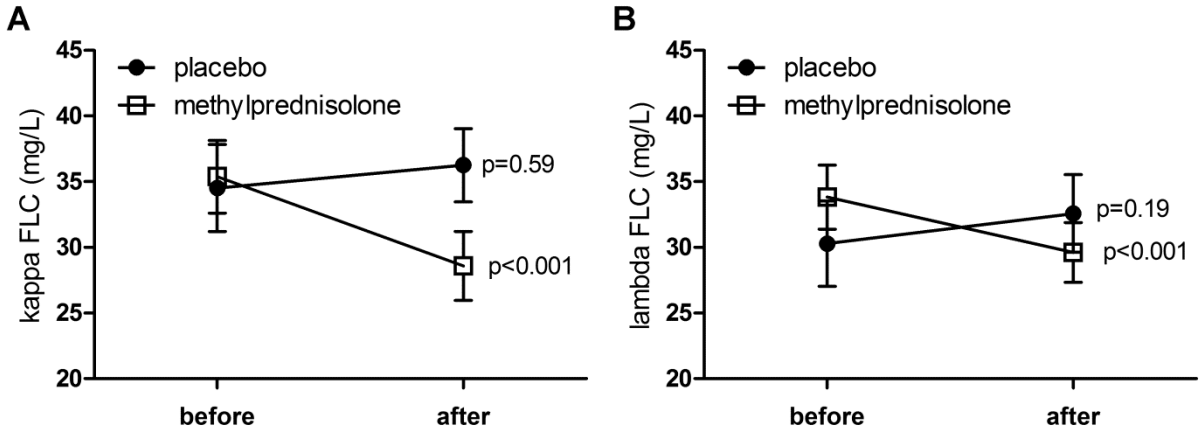

Figure 4. Serum κ-FLC (**A**) and λ-FLC (**B**) concentrations significantly decrease in patients with CRSwNP after 2 weeks treatment with methylprednisolone. Placebo treatment did not influence serum FLC concentrations. Methylprednisolone treatment did not influence local FLC concentrations in nasal secretions.

Mepolizumab treatment significantly reduces local FLC concentrations in patients with severe nasal polyposis

Anti-IL-5 treatment (mepolizumab) significantly reduced both κ- and λ-FLC concentrations in nasal secretions (κ-FLCs: before: 18.3±3.02; after: 12.2±4.17, p=0.05; λ-FLC: before: 15.8±3.76; after: 9.11±3.59, p=0.03). In this study placebo treatment did not affect nasal FLC concentrations (κ-FLCs: before: 28.6±9.33; after: 47.3±17.76, p=0.65; λ-FLC: before: 23.0±6.2; after: 44.0±18.09, p=0.91) (Figure 5A-B). Serum FLC concentrations were unaffected by mepolizumab. Serum and nasal total IgE did not significantly changed after treatment.[26] Correlations between changes in FLC concentrations and treatment effect were not found. Moreover, baseline FLC concentrations in polyp tissue homogenates did not predict treatment outcome.

Discussion

In this study the presence of FLCs in CRSwNP and CRSsNP patients was investigated and the effect of different treatments on FLC expression was analyzed. CRS patients had highly increased FLC concentrations in mucosal tissue homogenates and nasal secretions and this effect was most prominent in CRSwNP patients. These increased FLC concentrations were confirmed by immunohistochemical analysis of polyp tissue. Interestingly, local FLC concentrations are reduced in CRSwNP patients after anti-IL-5 treatment. Together, these observations further support an ongoing local immune reaction in the paranasal cavities of CRSwNP and CRSsNP patients and indicate a role for IL-5 in creating an environment that favors FLC production.

CRSwNP in Caucasian patients is characterized by a Th2 mediated eosinophilic immune response with high IL-5, ECP and IgE concentrations, whereas CRSsNP is mainly a Th1 immune response with high levels of IFN-γ and TGF-β.[4, 12, 35, 36] Highest FLC concentrations were found in tissue homogenates and nasal secretions from CRSwNP patients, which is in line with high local IgE, IgA and IgG concentrations and increased CD19+ B cells and plasma cell numbers in NP tissue.[4, 37] Moreover FLC concentrations in CRSwNP tissue are correlated to IL-5 and local IgE. This polyclonal IgE production is independent of atopic status and, in addition, *Staphylococcus aureus* is an important activator of this IgE production.[7, 8] Yet less pronounced, FLC concentrations were also increased in mucosal tissue from CRSsNP patients and correlated to MPO concentrations.

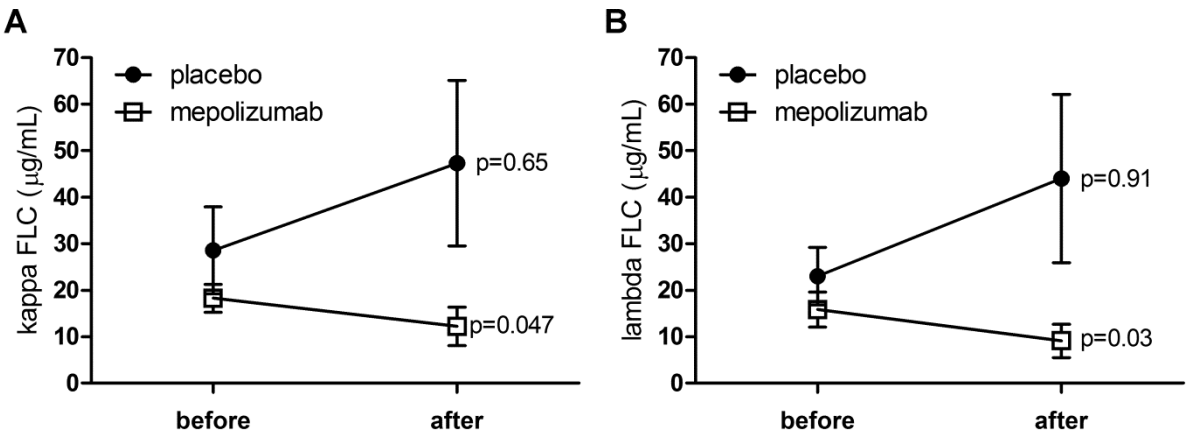

Figure 5. κ-FLC (**A**) and λ-FLC (**B**) concentrations in nasal secretions significantly decrease in patients with severe nasal polyposis 8 weeks after treatment with mepolizumab (2 single IV injections; 4 weeks interval). Placebo treatment did not influence serum FLC concentrations. Mepolizumab treatment did not influence systemic FLC concentrations.

Based on these findings it appears that local FLC production is not specifically associated to either a Th1 or Th2 mediated inflammatory response. This is in line with previous findings

showing increased FLC production in both IgE- and non-IgE mediated disorders, including asthma and rhinitis.[20, 21] Comparable to most CRSwNP cases,[2, 6, 38] eosinophilic infiltration of the nasal mucosa is characteristic for nonallergic rhinitis, especially in nonallergic rhinitis with eosinophilia syndrome (NARES) patients.[39-41] In this latter group, we previously showed that FLCs are highly increased in nasal secretions.[20] Moreover, the number of FLC-positive cells in nasal mucosa including mast cells and plasma cells, as detected by laser microdissection and subsequent RT-PCR analysis, were increased in both IgE- and non-IgE mediated rhinitis patients as compared to controls. Both CRSwNP and NARES are characterized by a tissue eosinophilia. Therefore, FLCs might be complementary actors in both disorders next to tissue eosinophilia.

Previously, preclinical models showed that FLCs might be involved in different inflammatory disorders such as contact hypersensitivity and asthma by mediating antigen-specific mast cell activation.[19, 21] Since mast cell involvement is implicated in CRSwNP, we analyzed FLC expression in nasal polyp tissue. However, in contrast to the cell-localized FLC staining pattern observed in nasal mucosa from rhinitis patients,[20] we observed a highly diffuse FLC staining in NP tissues that hampered the identification of possible low/moderate FLC-positive cells. Nevertheless, staining brightness correlated with nasal FLC concentrations as measured by ELISA and isotype controls showed negative results, indicating specificity of this staining.

So far, these data do not prove a functional role for FLCs in mediating NP pathology. Nevertheless, we and others have shown that FLC are able to specifically bind antigen.[19, 42, 43] Since CRSwNP pathology is highly associated to *Staphylococcus aureus* infection and presence of *Staphylococcus aureus* enterotoxin (SAE) specific IgE,[8] together with the finding that *ex vivo* cross-linking of IgE in nasal polyp tissue leads to mast cell mediated early phase-like responses,[14] it is interesting to analyze antigen binding capacity of nasal FLCs towards SAE in future studies. The finding that combined crosslinking of IgE and FLC resulted in a synergistic mast cell activation in *in vitro* assays,[44] further substantiates a putative functional role for FLCs in local mast cell activation in NP tissue. Recently, the therapeutic effect of oral glucocorticosteroids and antibiotics (methylprednisolone and doxycycline, respectively) was investigated in a double blind randomized manner, demonstrating a reducing nasal polyp size compared to placebo.[27] In line with the lack of a clear decrease of local IgE concentrations after these treatments, nasal FLC concentrations were not affected. We did find a highly significant decrease in serum FLC concentrations after methylprednisolone treatment, which could be related to its potent systemic anti-inflammatory action. This effect was absent in doxycycline treated patients, despite the ability of doxycycline to inhibit immunoglobulin production.[45]

IL-5 is highly expressed in nasal polyp tissue and this cytokine likely plays a critical role in chemotaxis, activation and survival of eosinophils.[4, 46] Furthermore, in this study FLC concentrations were correlated to tissue IL-5 levels in CRSwNP. It is proposed that eosinophils highly contribute to nasal polyp growth by the release of toxic mediators.[6] In accordance with these findings, anti-IL5 treatment with reslizumab induced a decrease of eosinophil count in serum and of ECP levels in nasal secretions.[47] Moreover mepolizumab, a humanized monoclonal anti-IL5 antibody, recently showed to be effective in reducing severe nasal polyposis in 12 out of 20 patients.[26] In the present study we observed a clear decrease in nasal local FLC concentrations after anti-IL5 treatment, whereas nasal IgE and serum IgE and FLC concentrations were unaffected [26]. Even though clear effects of IL-5 on B cells function are described,[48] it is unclear how blockage of IL-5 leads to a specific decrease in FLC production. The discrepancy between the effect of anti-IL-5 treatment seen on IgE and FLC concentrations, suggest that different mechanisms are responsible for their local expression. Alternatively, this finding could be explained by the existence of cells that appear to selectively produce FLC instead of complete immunoglobulins is also observed in NARES patients as described above,[20] and in idiopathic pulmonary fibrosis patients who showed little IgE and IgG in their BAL fluid though highly increased FLC concentrations were observed.[49] Clear regulatory mechanisms involved in FLC production by B cells are lacking though it has been demonstrated that immature B cells are mainly responsible for FLC production.[50-52]

In summary, FLCs are highly present in the nasal mucosa and nasal polyp tissue of CRSsNP and CRSwNP patients, respectively, emphasizing their role at the local tissue level. However, the exact functional role of the increased FLC expression at the tissue level, including their potential role in mediating local mast cell activation, needs to be further investigated. Methylprednisolone and doxycycline treatment of CRSwNP patients did not reduce local FLC concentrations, although serum concentrations significantly decreased after corticosteroid treatment. In contrast, anti-IL-5 treatment of severe CRSwNP patients decreased nasal FLC concentrations. Environmental conditions that influence FLC production are still largely unknown and the influence of IL-5 on this process observed in this study warrants further research. Finally, future studies need to address putative effects of FLC on eosinophils function.

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5

Immunoglobulin free light chains are increased in hypersensitivity pneumonitis and idiopathic pulmonary fibrosis

This chapter is published in *PLoS ONE*, 2011;6(9) e25392.

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Abstract

Background: Idiopathic pulmonary fibrosis (IPF), a devastating lung disorder of unknown aetiology, and chronic hypersensitivity pneumonitis (HP), a disease provoked by an immunopathologic reaction to inhaled antigens, are two common interstitial lung diseases with uncertain pathogenic mechanisms. Previously, we have shown in other upper and lower airway diseases that immunoglobulin free light chains (FLCs) are increased and may be involved in initiating a local inflammation. In this study we explored if such mechanism may also apply to HP and IPF.

Methods: In this study we examined the presence of FLC in serum and BAL fluid from 21 IPF and 22 HP patients and controls. IgG, IgE and tryptase concentrations were measured in BAL fluid only. The presence of FLCs, plasma cells, B cells and mast cells in lung tissue of 3 HP and 3 IPF patients and 1 control was analyzed using immunohistochemistry.

Results: FLC concentrations in serum and BAL fluid were increased in IPF and HP patients as compared to control subjects. IgG concentrations were only increased in HP patients, whereas IgE concentrations were comparable to controls in both patient groups. FLC-positive cells, B cells, plasma cells, and large numbers of activated mast cells were all detected in the lungs of HP and IPF patients, not in control lung.

Conclusion: These results show that FLC concentrations are increased in serum and BAL fluid of IPF and HP patients and that FLCs are present within affected lung tissue. This suggests that FLCs may be involved in mediating pathology in both diseases.

Introduction

Interstitial lung diseases (ILD) comprise a diverse group of disorders affecting the lung parenchyma that are classified together because they share similar clinical, radiographic, and physiologic features [1]. Two frequent and complex ILD are idiopathic pulmonary fibrosis (IPF) and hypersensitivity pneumonitis (HP).

IPF is a chronic fibrosing interstitial pneumonia of unknown aetiology limited to the lungs and associated with the histopathologic pattern of usual interstitial pneumonia (UIP)[2]. It is characterized by alveolar epithelial cell injury and activation, expansion of the fibroblast/myofibroblasts population forming the so called fibroblastic foci and the exaggerated accumulation of extracellular matrix [3,4]. The disease is usually progressive and does not have effective therapy [5]. Hypersensitivity pneumonitis consists of a group of lung disorders resulting from exposure to a wide variety of organic particles causing an immunopathological reaction of the lungs in susceptible individuals [6]. One of the most frequent aetiologies of HP is the inhalation of bird-derived proteins that provoke the so-called pigeon breeders' disease (PBD). The clinical behavior is heterogeneous and may present as acute, sub-acute or chronic forms, often with overlap between these interrelated categories [7]. Importantly, patients with chronic HP may evolve to interstitial fibrosis, and in advanced stage may be very difficult to distinguish from IPF/UIP [8,9]. Strong evidence indicates that sub-acute and chronic HP is primarily a T-cell mediated hypersensitivity [10]. Less is known about B lymphocyte involvement, although some participation is suggested by the antibody response to inhaled antigens resulting in high titers of circulating specific antibodies and the presence of plasma cells in the bronchoalveolar lavage mainly in sub-acute cases [11,12].

Mast cell involvement in ILD pathology is uncertain but it is shown that increased numbers of mast cells are present in bronchoalveolar lavage (BAL) fluid of both IPF and HP patients [11,13-17]. Moreover, these mast cells show activated phenotypes, the mast cell products histamine and tryptase are detectable in BAL fluid, and mast cell counts in lung biopsies positively correlate with the degree of fibrosis [15,18]. Interestingly, mast cells can be rich sources of profibrotic cytokines, growth factors and proteases which are known to modulate the fibrotic process like transforming growth factor-β (TGF-β), IL-1, IL-4, IL-13, tumor necrosis factor-α (TNF-α), chymase, and tryptase [14,19-21]. Furthermore, mast cells can produce a plethora of mediators involved in the recruitment and activation of other inflammatory cell types like lymphocytes and monocytes.

Previously we have shown that immunoglobulin free light chains (FLCs) can mediate antigen-specific mast cell activation [22]. FLC concentrations are increased in different immune disorders in which mast cells appear to play a prominent function like rheumatoid

arthritis, inflammatory bowel disease, and multiple sclerosis, and some respiratory disorders like asthma and rhinitis [23-26]. The aim of this study was to investigate FLC expression in IPF and HP patients, and relate these findings to immunoglobulin concentrations, inflammatory cells present in affected lungs, and pulmonary function tests. Furthermore, the number of mast cells and its activation state was analyzed in both patient groups and compared to controls.

Patients and Methods

Study population

Blood and BAL samples were obtained from 21 patients with IPF and 22 patients with chronic HP induced by exposure to avian antigens (pigeon breeders' disease). None of the patients had been treated with corticosteroids or immunosuppressive drugs at the time of the study. As controls, blood samples and BAL fluids were achieved from 11 and 4 healthy individuals respectively. The study was approved by the Bioethics committee at the National Institute of Respiratory Diseases, and informed consent was obtained from all subjects.

Diagnosis of IPF was performed according to the American Thoracic Society/European Respiratory Society consensus [27]. Open lung biopsy was performed in 46% of the patients and all of them showed typical microscopic findings of usual interstitial pneumonia [28]. In the absence of biopsy, patients had to fulfil the criteria of the ATS/ERS international consensus, including a confident HRCT scan [29]. Diagnosis of chronic HP was obtained as described elsewhere [10] and based on the following criteria: a) history of pigeon exposure and positive serum antibodies against avian antigens; b) clinical, radiological, and functional features of an ILD with $\geq$ 6 months of symptoms; c) >30% lymphocytes in BAL fluid; and d) lung histology compatible with HP.

Bronchoalveolar lavage

BAL was performed through flexible fiberoptic bronchoscopy under local anaesthesia as previously described [30]. Briefly, 200 ml of normal saline was instilled in 50-ml aliquots, with an average recovery of 60%-70%. The recovered BAL fluid was centrifuged at 250 g for 10 min at 4 °C. The cell pellet was resuspended in 1 ml of phosphate buffered saline (PBS) and an aliquot was used to evaluate the total number of cells. Other aliquots were fixed in carbowax, stained with hematoxylin and eosin, and used for differential cell count. Supernatants were kept at -70°C until use.

Total FLC, IgE, IgG, and tryptase measurement

Total serum or BAL fluid kappa (κ) and lambda (λ) FLC concentrations were determined using an ELISA adapted from Groot Kormelink *et al* [23]. In brief, plates were coated (o/n; 4^{o}C) with goat anti-mouse IgG (M4280, Sigma, Zwijndrecht, The Netherlands) and subsequently blocked (1 hour; RT) and incubated with mouse anti- human kappa (κ) or lambda (λ) Ig-FLC MAb's (obtained from Dr. A. Solomon, Tennessee, US). After incubation with different dilutions of samples and standards (Binding Site, Birmingham, UK), plates were incubated with HRP-labelled goat F(ab')$_2$-anti human kappa or lambda immunoglobulin light chain antibodies (AHI1804 and AHI1904, respectively, Biosource, USA). TMB was used as a substrate. Per sample, at least three data points within the range of the standard curve were used to estimate the FLC concentration. For measurements of total IgE, total IgG and tryptase in BAL fluid the ImmunoCAP 100® system (Phadia AB, Uppsala, Sweden) was used. For total IgG, BAL samples were pre-diluted 100 times in specific IgA/IgG sample diluent. All tests were performed according to the manufacturer's instructions. Total IgE concentrations are expressed in kU/L, total IgG concentrations in mgA/L and tryptase concentrations in µg/L. Total IgE antibody concentrations ≥ 0.35 kU/L, total IgG antibody concentrations ≥ 0.07 mgA/L, and tryptase concentrations ≥ 1 ug/L were considered positive.

Immunohistochemistry

Immunohistochemistry was performed on 4 µm thick formalin-fixed, paraffin embedded lung tissue sections from 5 IPF, 5 HP subjects and 3 controls. In short, serial tissue sections were subjected to heat induced antigen retrieval for 25 minutes (S1700, Dakocytomation, Haverlee, Belgium), blocked with PBS-T/3%BSA/3% normal goat serum for 1 hour, and incubated (over night) with the following primary antibodies diluted in blocking buffer: mouse-anti-human kappa FLC (Fκ-C8) and mouse-anti-human lambda FLC (Fλ-G9) (both obtained from Dr. A. Solomon, Tennessee, USA), mouse-anti-human CD138 (clone MI15, Dakocytomation, Haverlee, Belgium), rabbit-anti-human CD20 (clone BV11, Abcam,Cambridge, UK), and biotin-conjugated mouse-anti-human tryptase (G3361, Promega, Leiden, The Netherlands). Tissues were subsequently incubated with Alexa Fluor 568 goat-anti-mouse IgG, Alexa Fluor 488 goat-anti-rabbit IgG, or streptavidin Alexa Fluor 568 conjugate (all Invitrogen, Breda, The Netherlands) for 1 hour. Nuclei were counterstained with diamidino-phenylindole (DAPI, Invitrogen, Breda, The Netherlands). For mast cell number quantification, a mean number of mast cells per patient was calculated by averaging the number of mast cells counted in seven randomly taken pictures (200x magnification) per tissue. Sections were viewed with an Eclipse TE2000-U inverted

Table 1. Demographic and clinical characteristics of the patients with hypersensitivity pneumonitis and idiopathic pulmonary fibrosis.

	HP (n=22)	*IPF (n=21)*	*Control (serum; n=11)*	*Control (BAL; n=4)*
Age, yr	52 ±12	66 ±8	42±20	53±15
Sex (M/F)	4/18	14/7	2/9	3/1
Current or former smoking	3/22	11/21	2/9*	1/4
Bronchoalveolar Lavage				
Macrophages, %	48 ±19	84 ±10		
Lymphocytes, %	50 ±19	12 ± 7		
Neutrophils , %	0.8 ±0.9	2.4 ±3.4		
Eosinophils, %	1.2 ±2	1.8 ±2.9		
Pulmonary Function Tests				
DLCO, % predicted	46 ±20	42 ±18		
FVC, % predicted	61 ±16	63 ±24		
FEV1, % predicted	64 ±18	68 ±26		
SpO2, % at rest	88 ±4	87 ±5		
SpO2, % during exercise	75 ±19	81 ±6		
PaO2 mm Hg**	54.8 ±7.2	56.2 ±9		
ePSAP mm Hg	31 ±18	48 ±18		

Mean values are shown ± standard deviation.*=unknown for 2 controls; **PaO2 = arterial pressure oxygen; normal values at Mexico City altitude are 67±3 mmHg. SpO2= pulse oxygen saturation. FEV1= volume of air expired during the first second. FVC= forced vital capacity. DLCO= carbon dioxide diffusing capacity. ePSAP= estimated systolic pulmonary artery pressure.

microscope (Nikon) or a Zeiss LSM-510 confocal microscope. Images were analyzed using NIS elements BR 2.3 (Nikon) or Zen 2007 (Zeiss) software, respectively.

Statistical analysis

FLC, IgG and IgE concentrations in serum and BAL fluid were compared using a One-Way ANOVA. Subsequently, a post-hoc Dunn's multiple comparison test was performed on all patients groups. Correlations between all serum and BAL fluid parameters were determined by Spearman correlation coefficient. Tissue mast cell numbers were analyzed using a One-Way ANOVA followed by a bonferroni multiple comparison test. P Values were considered significant when $p<0.05$. All analyses were performed using GraphPad Prism, version 4.03.

Results

Demographic and clinical features

Demographic and clinical features of the analyzed patient groups are shown in table 1. All patients exhibited clinical, radiologic, and functional evidence of ILD, with variable degrees of dyspnoea, decreased lung volumes, and hypoxemia at rest that worsened during exercise.

Although the analyzed patient groups show considerable differences in age FLC concentrations are not much influenced by age in adults [31-33]. Furthermore, there is a difference in ratio of smokers/non-smokers between the groups but the 2 smoking controls had intermediate serum concentrations, suggesting that smoking does not significantly influence serum FLC.

Increased serum and BAL fluid FLC concentrations in IPF and HP patients

In serum and BAL fluid, FLC concentrations are significantly increased in HP and IPF patients as compared to healthy controls. Mean (±SEM) κ-FLC serum concentrations were 55.9±12.32 mg/L in HP, 54.7±4.04 mg/L in IPF, and 22.1±2.93 mg/L in control subjects (Figure 1A). For λ-FLC serum concentrations were 46.8±7.29 mg/L in HP, 46.6±3.44 mg/L in IPF and 18.3±3.02 mg/L in the control group (Figure 1B). Mean (±SEM) FLC concentrations in BAL fluid were 0.94±0.12, 1.11±0.20, 0.12±0.060 mg/L (κ-FLC) and 0.45±0.07, 0.73±0.17, 0.06±0.03 mg/L (λ-FLC) in the HP, IPF and control group, respectively (Figure 1C and D). FLC concentrations in BAL fluid showed no significant correlation with those in serum.

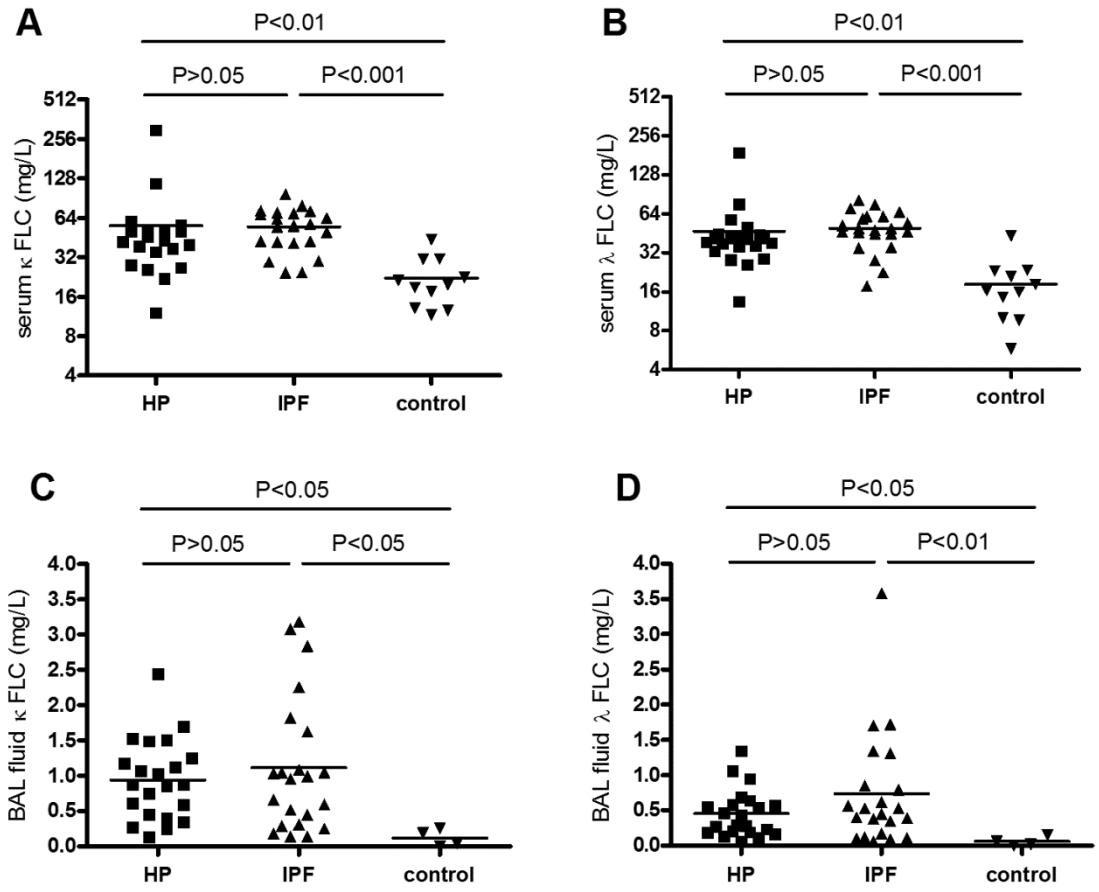

Figure 1. Free light chain concentrations in serum and BAL fluid from HP and IPF patients. Kappa and lambda FLC concentrations are highly increased in serum (**A** and **B**, respectively) and BAL fluid (**C** and **D**, respectively) of the HP and IPF groups compared with control subjects. Mean FLC concentrations are similar in the IPF and HP groups.

Total IgG, IgE and tryptase concentrations in BAL fluid from HP and IPF patients

Both IgG and IgE antibodies were detectable in BAL fluid from HP and IPF patients and control subjects. The mean (±SEM) total IgG concentration in HP patients (29.8±5.58 mgA/L) was significantly higher than that in IPF patients (11.6±2.28 mgA/L) and control subjects (5.3±3.70 mgA/L) (both $p<0.05$). Total IgG concentrations in BAL fluid from IPF patients and controls did not differ significantly (Figure 2A). Total IgE concentrations were similar in all groups (Figure 2B). Tryptase was only detectable in 2 patients, one HP patient (9.27 µg/L) and one IPF patient (2.27 µg/L) (data not shown).

Correlations between BAL fluid humoral factors and clinical parameters

In the HP and IPF patient groups we investigated the relationship between FLC and BAL fluid IgG concentrations and inflammatory cell numbers, and the clinical parameters as described in table 1. For FLC, a positive correlation was found between λ-FLC and DLCO% in the HP patient group ($p=0.0021$; $r=0.62$). A positive correlation was also found between total IgG concentrations and the number of BAL fluid lymphocytes in HP ($p=0.006$; $r=0.57$) and IPF ($p=0.037$; $r=0.46$) patients. A significant positive correlation between IgG and kappa and lambda FLC was found in IPF patients (IgG vs κ-FLC: $p=0.009$; $r=0.55$; IgG vs λ-FLC: $p=0.045$; $r=0.44$).

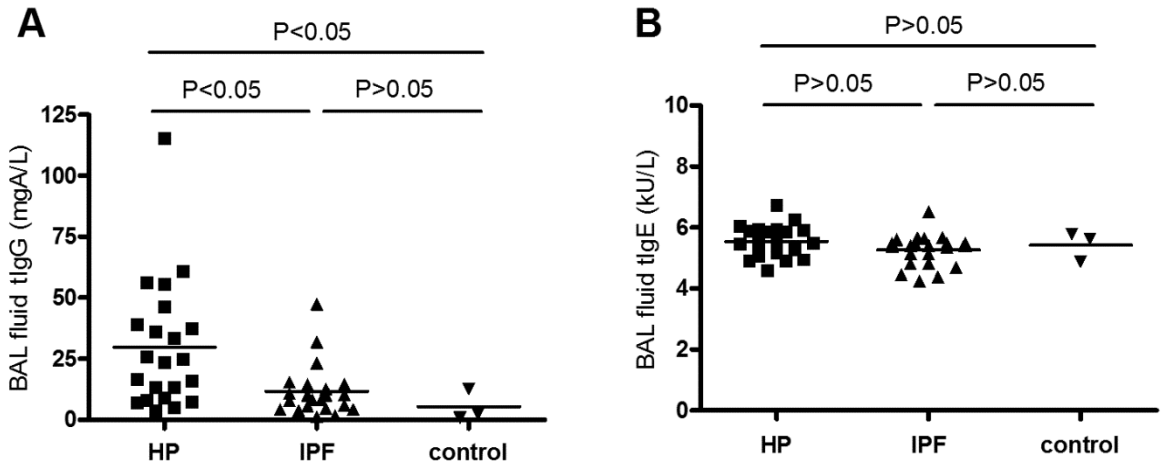

Figure 2. IgG and IgE concentrations in BAL fluid from HP and IPF patients. Total IgG concentration in BAL fluid was increased in the HP but not in the IPF and control group (**A**). Total IgE concentrations in BAL fluid were similar in the HP, IPF, and control group (**B**).

FLC-positive cells, B cells, plasma cells and mast cells in lung tissue from HP, IPF and control subjects

Immunohistochemical analysis of lung tissue from all HP and IPF patients revealed similar staining patterns for FLC, CD20 (B-cells), CD138 (plasma cells), and tryptase (mast cells).

Many FLC-positive cells were detected throughout the tissue as isolated cells or in small groups (Figure 3A). Numerous B cells and plasma cells were detectable, either as single cells or clustered in groups and in vicinity of each other (Figure 3B). B cells, and plasma cells were not detectable in control lung tissues, and FLC-positive cells were here only detected scarcely. Tryptase positive cells were present throughout all lung tissues, including control tissue. However, the number and density of mast cells was lower in the control tissue (Figure 4A) and morphological differences were dramatically apparent between mast cells in HP and IPF lungs as compared to mast cells in healthy control lungs (Figure 4B-E). Mast cells in control tissue had a non-activated phenotype (Figure 4B and D), whereas many mast cells in HP and IPF lungs showed signs of activation and degranulation (Figure 4C and E).

Discussion

In the present study, we show that FLCs are increased in serum and BAL fluid from IPF and HP patients as compared to controls. Furthermore, FLC-positive cells are present in the lungs of IPF and HP patients. Total IgG concentrations were only increased in HP patients, whereas total IgE concentrations in IPF and HP patients were not different from healthy controls.

The pathological mechanisms involved in IPF and HP are yet unclear, but the putative contribution of mast cells in mediating fibrosis in both disorders has been recognized [15,18,34]. The high number of mast cells and their activated appearance in the lungs of IPF and HP patients compared to normal lung tissue observed in this study supports previous observations indicating mast cell involvement in both disorders. Interestingly, increased tissue or BAL fluid mast cell numbers were shown to correlate with the degree of fibrosis and activity of disease [11,16,17,35]. Moreover, tryptase was shown to be present in BAL fluid from IPF and HP patients, supporting the morphological features of mast cell activation [18]. In this study however, we only found detectable concentrations of tryptase in BAL fluid from 2 of 44 patients. Because histological analysis of mast cells in lung tissue from IPF and HP patients clearly demonstrated their activated appearance, timing and location of sampling and/or analytical detection limits may explain the discrepancy with the BAL tryptase analysis.

Despite the activated mast cell appearance, a possible activation mechanism in IPF and HP lungs is not elucidated. The involvement of IgE in IPF and HP is not likely, because IgE concentrations in BAL fluid are not increased compared to healthy controls. Many other stimuli such as FLC, IgG, complement factors, toll-like receptor ligands, and bacterial and viral products can induce mast cell activation [22,36]. In agreement with other studies, we found increased concentrations of total IgG in BAL fluid from HP patients and specific IgG

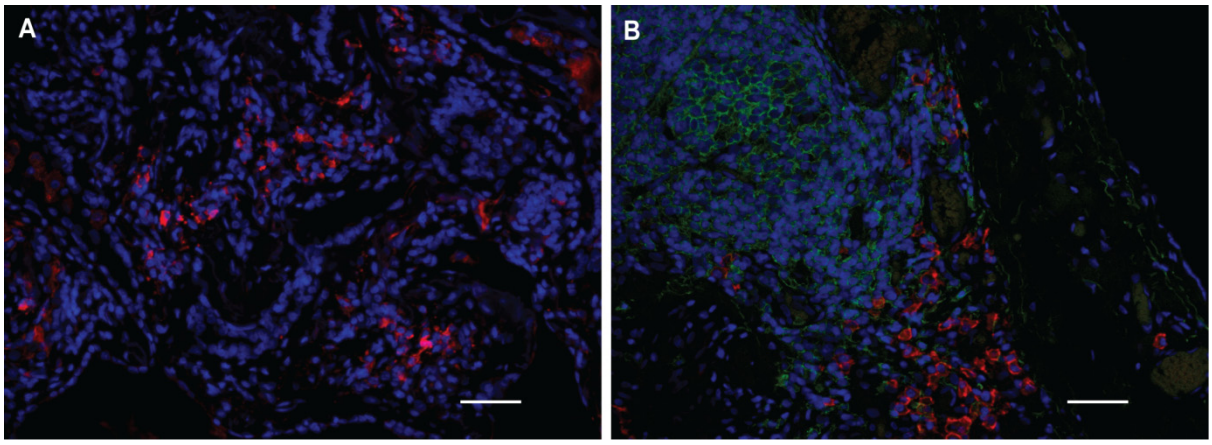

Figure 3. FLC-positive cells, B cells, and plasma cells in the lungs of HP and IPF subjects. Representative pictures are shown of numerous FLC-positive cells (both kappa and lambda FLC are stained red) (**A**), and B cells (green) and plasma cells (red) (**B**) which are found in HP and IPF lung tissue. Scale bar: 50µm.

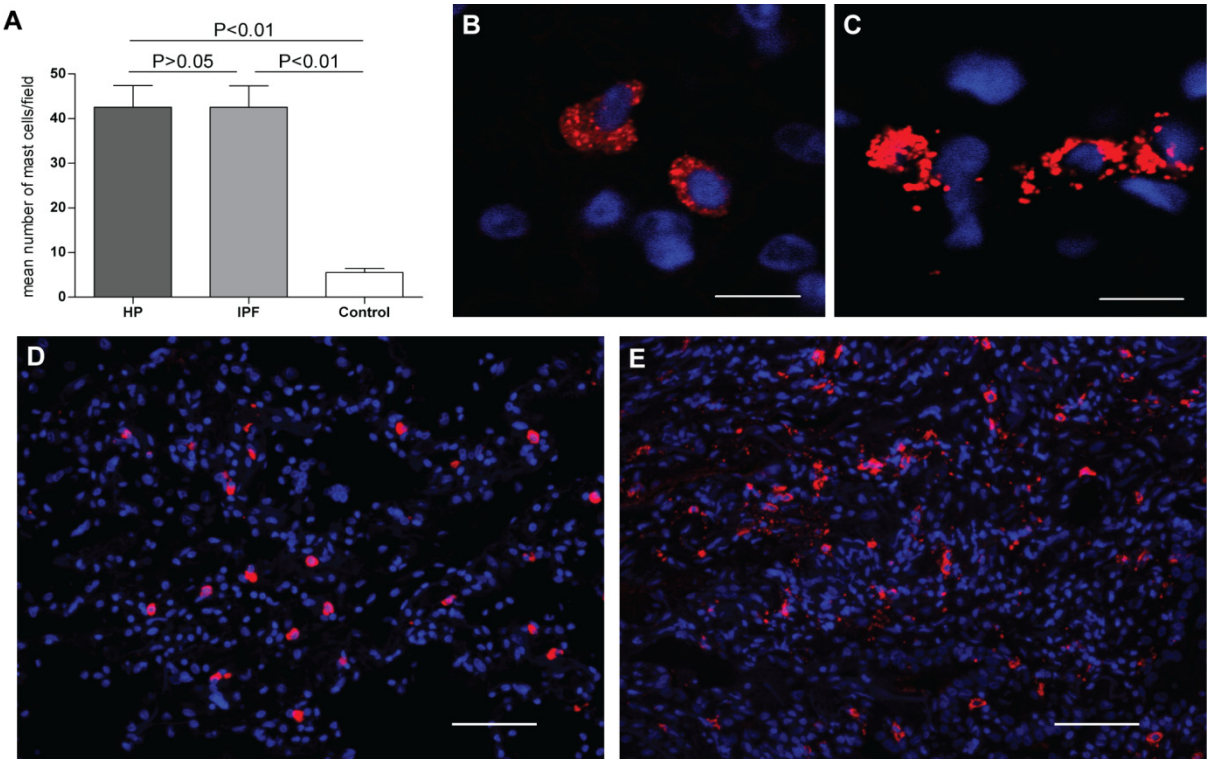

Figure 4. Numerous mast cells are found in the lungs of HP, IPF and control subjects. The number of mast cells is significantly higher in HP and IPF patients compared to controls (**A**). Per patient, the mean number of mast cells is calculated by averaging the number of cells counted in seven randomly taken pictures of the tissue. Bars represent the means ± SEM for 5 IPF and 4 HP patients and 3 controls. Representative pictures are shown of tryptase positive mast cells (red) with a non-activated phenotype as mainly found in control tissue (**B** and **D**), and tryptase positive mast cells with an activated/degranulated phenotype as mainly found in HP and IPF tissue (**C** and **E**). **B** and **C** are representative confocal images taken from tissue shown in **D** and **E**, respectively. Scale bar **B,C**: 10µm; **D,E**: 50µm.

against the sensitizing antigen have been demonstrated in HP lungs [12]. This suggests that IgG could be involved in triggering mast cell activation in lungs of HP patients. Alternatively FLCs, which we found significantly increased, could mediate antigen-specific mast cell activation [22]. Thus, together, FLCs and IgG might provide a mechanism by which antigen-specific mast cell activation takes place in the lungs of HP patients. In our previous work we have demonstrated a crucial role of FLC in experimental models for allergen-induced contact hypersensitivity, asthma, food allergy and IBD [22,24,25]. Whether antigen specific FLC can be detected in human body fluids is the subject of current research.

IPF is not an antigen-specific mediated disease, but viral infections seem to be common in these patients [37,38] and although IPF is a multi-factorial disease, a growing body of evidence implicates viruses as co-factors, either as initiating or exacerbating agents [39]. Viral infections increase the occurrence of FLCs and interestingly, we have recently shown that FLC concentrations are greatly increased during viral myocarditis in mice and that FLC may play a protective role in the pathogenesis of disease [40].

The presence of FLCs in BAL fluid together with the high number of B cells and plasma cells in lung tissue in most IPF and HP patients supports the concept of local production of immunoglobulins in the lungs as suggested earlier for HP patients [11]. In IPF, primarily in areas of honeycombing changes (advanced disease), small lymphoid aggregates formed by B and T cells are usually noticed [41]. Likewise, B cells and plasma cells are seen within the lumen of bronchioles and alveolar walls [12]. The fact that FLC concentrations in serum are also increased might be caused by an overspill of locally produced FLC into the circulation. For example, such systemic increases in FLC have also been observed in asthma patients [25]. The low FLC concentrations detected in BAL fluid from controls are in line with the absence of plasma cells in healthy lung parenchyma [42].

FLCs and IgG concentrations appear not to be prognostic biomarkers for lung function capacity, since no clear correlations were found with different physiological and functional parameters. This lack of correlation impedes a conclusion on a functional role of FLCs in disease pathology, which could be due to the low number of patients per disease group available for our analysis. Furthermore, patients were in a medium/late stage of disease and this late phase in disease progression might hamper the detection of a functional role of FLCs in the initiation phase of disease. In addition, the effect of therapeutic treatment on the expression of FLCs is unexplored.

In conclusion, this study is the first to show the increased presence of FLCs in serum and bronchoalveolar lavage fluids from IPF and HP patients. Furthermore, in lung tissue from patients of both types of disease, many B cells, plasma cells, and activated mast cells can

be found. These findings suggest that possible common immunologic mechanisms may be involved in both diseases, despite the differences in pathogenesis.

In former studies we described that antigen-specific FLC-mediated mast cell activation can regulate inflammatory responses in mice. This study shows presence of FLCs in HP and IPF, but whether they contribute to disease pathology via mediating mast cell activation remains presently unknown. Future research should also disclose if cell types other than mast cells known to be important in IPF and HP, like fibroblasts, macrophages, and neutrophils, are affected by FLC and whether prevention of FLC-induced activation of immune cells could be of therapeutic value in the treatment of HP and IPF.

Acknowledgments

We thank Dr. G. Posthuma (Cell Microscopy Center, Dept of Cell Biology, UMCU, Utrecht, The Netherlands) for his help with the Zeiss LSM-510 confocal microscope and Dr. Amit Gaggar (University of Alabama, USA) for his assistance to initiate these studies.

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6

Decrease in immunoglobulin free light chains in patients with rheumatoid arthritis upon rituximab (anti-CD20) treatment correlates with decrease in disease activity

This chapter is published in *Annals of the Rheumatic Diseases*, 2010;69:2137-44

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Abstract

Objectives: Immunoglobulin (Ig) free light chains (FLCs) are short-lived B cell products that contribute to inflammation in several experimental disease models. In this study, FLC concentrations in inflamed joints of patients with rheumatoid arthritis (RA) as compared to patients with osteoarthritis were investigated. In addition, the relationship of FLCs and disease activity upon B cell depletion (rituximab) in patients with RA was studied.

Methods: Synovial fluid (SF) and tissue from patients with RA were analyzed for local presence of FLCs using ELISA and immunohistochemistry. In addition, FLC concentrations were measured (at baseline, 3 and 6 months after treatment) in 50 patients with RA with active disease who were treated with rituximab. Changes in FLCs were correlated to changes in disease activity and compared to alterations in IgM, IgG, IgA, IgM-rheumafactor (RF), and IgG-anti-citrullinated protein antibody (ACPA) concentrations.

Results: FLCs were detected in synovial tissue from patients with RA, and high FLC concentrations were found in SF from inflamed joints, which positively correlate with serum FLC concentrations. Serum FLC concentrations significantly correlated with disease activity score using 28 joint counts, erythrocyte sedimentation rate (ESR), and C reactive protein, and changes in FLC correlated with clinical improvement after rituximab treatment. Moreover, effect of treatment on FLC concentrations discriminated clinical responders from non-responders, whereas IgM-RF and IgG-ACPA significantly decreased in both patient groups.

Conclusions: FLCs are abundantly present in inflamed joints and FLC levels correlate with disease activity. The correlation of FLC concentrations and disease activity indicates that FLCs may be relevant biomarkers for treatment response to rituximab in patients with RA and suggests that targeting FLC may be of importance in the therapy of RA.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disorder featured by inflammation of synovial tissue, characterized by proliferation of synoviocytes, production of pro-inflammatory mediators and subsequent infiltration of predominantly mononuclear cells.[1 2] In chronically inflamed synovial tissue, macrophages, T cells, B cells, plasma cells and mast cells are abundantly present. Although RA has often been considered predominantly a T cell mediated macrophage-dependent disease, [3 4] the importance of B cells and their products have been well recognized at present.[5] Administration of B cell depleting antibody directed to CD20 (rituximab) to patients with RA results in profound and longstanding depletion of peripheral CD20+ B cells in most patients. In a significant number of patients, this leads to a decrease in disease activity.[6 7] Several mechanisms through which B cells can contribute to inflammation are proposed, like antigen presentation to T cells, and production of proinflammatory cytokines, chemokines and autoantibodies (eg, rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA)),[8] which can trigger immune complex-mediated responses.[9 10] Although these autoantibodies decline after rituximab treatment, this does not consistently correlate with clinical response.[11 12] Since a clear understanding of the working mechanism of rituximab is lacking, it cannot be excluded that B cell products other than cytokines and autoantibodies are important in mediating disease at the local and systemic level. One of these putative other factors are κ and λ immunoglobulin (Ig) free light chains (FLCs). FLCs are produced physiologically by B lymphocytes, plasmablasts and/or plasma cells. It has been shown that FLCs can exhibit several biological activities like enzymatic activity, complement activation, specific binding activity to antigens, and binding to different cell types, including mast cells.[13] Serum FLC concentrations are demonstrated to be increased in several inflammatory diseases including asthma, rhinitis, multiple sclerosis, inflammatory bowel disease, and RA.[13-17] In RA, serum FLC concentrations correlate with disease activity.[14 18]

In the present study, we demonstrate increased local and systemic concentrations of FLC in patients with RA. In addition, we document that downregulation of FLCs by rituximab is associated with clinical improvement.

Patients and methods

Patients and treatment protocol

To measure local FLC concentrations in the joint, synovial fluid (SF) was collected from 68 randomly selected patients with clinical diagnosis of RA according to American College of Rheumatology (ACR) criteria,[19] and 24 patients with osteoarthritis (OA). Paired serum

samples were collected at the same time from a subgroup of these patients with RA (11 patients). To assess presence of FLCs in joint tissue, synovial tissue was collected from eight randomly selected patients with RA and patients with OA that underwent total knee joint replacement.

To measure the effect of rituximab treatment on serum FLCs, another 50 patients with RA were included in an open study with a follow-up of 6 months. Patients were eligible for enrolment if they were 18 years of age or older, had a clinical diagnosis of RA according to ACR criteria [19] and failed treatment with combination(s) of disease-modifying antirheumatic drugs and/or tumour necrosis factor (TNF)-blocking agents. Patients were excluded when one of the following criteria was present: life expectancy of less than 6 months, severe uncontrolled infections, irreversible major organ dysfunction, HIV positivity, a positive pregnancy test or unwillingness to use adequate contraception for the duration of the study. The patients were treated with two infusions of 1000 mg rituximab (Roche, Woerden, The Netherlands) at day 1 and day 14. Patients were assessed for disease activity using the Disease Activity Score in 28 joints (DAS28)[20] before the start of treatment, and 3 and 6 months after start of treatment. Efficacy end point was the response according to European Leage Against Rheumatism (EULAR) criteria.[21] At similar time intervals, blood was collected for the assessment of serum antibody titers, including FLCs, and the inflammatory parameters erythrocyte sedimentation rate (ESR) and C reactive protein (CRP). In addition, serum FLC concentrations were analyzed in a non-atopic control group (n=14). All patient material collection was performed according to the local Medical Ethical Committees of the University Medical Center, Utrecht and the Academic Medical Center, Amsterdam, and all patients gave their informed consent before participation in the study.

Measurements of serum antibody titers

Total serum immunoglobulin G (IgG), immunoglobulin M (IgM) and immunoglobulin A (IgA) titers were measured by immunoturbidimetry on the COBAS Integra 400/700/800 (Roche Diagnostics, Indianapolis, Indiana, USA) and nephelometry on the Immage 800 (Beckman Coulter Inc., Fullerton, California, USA) according to the manufacturer's guidelines. Serum titers of anti-cyclic citrullinated protein antibodies of the IgG isotype (IgG-ACPA) were measured using a commercial enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA, mark 2; Euro-Diagnostica, Arnhem, The Netherlands), according to the manufacturer's instructions and as previously reported.[22] [23] Serum titers of rheumatoid factor of the IgM isotype (IgM-RF) were measured using a standardized ELISA, as previously described.[23] [24]

Total serum or SF FLC concentrations were determined using an ELISA adapted from Abe *et al*.[25 26] (see supplementary material for details, [page 119]).

Immunohistochemistry.

Cryostat tissue sections were allowed to air dry at room temperature prior to tissue fixation using cold acetone for 10 min. After subsequent air drying and washing with PBS, sections were blocked with PBS-Tween/3% bovine serum albumin (BSA)/3% normal goat serum for 1 h, followed by overnight incubation with the following primary antibodies diluted in blocking buffer: mouse anti-human κ FLC (Fκ-C8) and mouse anti-human λ FLC (Fλ-G9) (both obtained from Dr. A. Solomon, University of Tennessee, Knoxville, USA), mouse anti-human CD138 (clone MI15, Dako Cytomation, Heverlee, Belgium), and rabbit anti-human CD20 (clone BV11, Abcam, Cambridge, UK). From each tissue, three serial sections were (double) stained for λ FLC and CD20, CD138, and κ FLC and CD20. After washing in PBS-T, tissue was incubated with Alexa Fluor 568 goat anti-mouse IgG or Alexa Fluor 488 goat anti-rabbit IgG (both Invitrogen, Breda, The Netherlands) for 1 h. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Sections were viewed with an Eclipse TE2000-U inverted microscope (Nikon, Lijnden, The Netherlands). Images were analyzed using NIS elements BR 2.3 software (Nikon).

Statistical analysis.

Differences in SF FLC concentrations between patients with RA and patients with OA were determined using a Mann-Whitney test for unpaired data. Correlations between serum and SF FLCs, serum FLC concentrations and ESR and CRP, and between changes in these parameters after treatment, were determined by Spearman correlation coefficient. Changes in the concentrations of FLC, IgM, IgM-RF and IgG-ACPA after treatment were analyzed by a one-way analysis of variance with repeated measures after log-transformation of the data. Sphericity was tested using Mauchly's test. Greenhouse-Geisser correction was applied when sphericity could not be assumed. Student t test was used to analyze whether different methotrexate or prednisone treatment dosages influenced FLC concentrations at baseline. p Values were considered significant when $P<0.05$. All analyses were performed using SPSS V.15 (SPSS, Chicago, Illinois, USA).

Results

Clinical and demographic features

Clinical and demographic features of all analyzed patient groups are shown in table 1.

FLCs are abundantly present in synovial fluid and synovial tissue of patients with RA

Serum FLC concentrations were significantly increased in patients with RA compared to healthy individuals. Using our standard ELISA, mean (±SEM) serum concentrations of healthy controls were 23.9±2.18 mg/L (κ) and 20.1±2.16 mg/L (λ) (data not shown), whereas concentrations in patients with RA were 56.3±6.17 and 53.5±5.31 mg/L, respectively (see below). SF FLC concentrations were highly increased in patients with RA, compared to patients with OA (figure 1A, mean ±SEM for κ and λ FLC concentrations in OA and RA were 24.6±2.37 and 161.2±33.2 mg/L, respectively, and 27.8±2.62 and 121.4±17.6 mg/L, respectively). Interestingly, maximum concentrations were as high as 1512 and 823.3 mg/L for κ and λ FLC, respectively.

Table 1. Baseline patient characteristics of analyzed patient groups

	RTX treated patients	Control group	RA (SF analysis[#])	OA (SF analysis)
Demographics				
Number of patients	50	14	67	24
Female, no (%)	36 (72)	7 (50)	49 (73)	15 (63)
Age, median (range) years	58 (22-84)	45 (23-73)	61 (26-80)*	62 (44-83)
Disease status				
Disease duration, median (range), years	12 (1-50)		19 (2-55)*	5 (2-34)
ESR, mean (±SD) mm/h	43.7 (±27.0)		50.8 (±40.7)*	NA
CRP, mean (±SD) mg/L	35.2 (±30.2)		47.8 (±65.3)*	NA
DAS28, mean (±SD)	6.5 (±1.1)		NA	NA
RF + (%)	41 (82)		41 (61)	
IgM-RF, mean (±SD) IU/mL	205.1 (±310.6)		NA	NA
IgG-ACPA, mean (±SD) IU/mL	1172 (±2268)		NA	NA
Medications				
No. of previous DMARDs, median (range)	5 (2-10)		NA	
No. of previous biological agents, median (range)	2 (0-4)		NA	
Biologics (aTNF n=9, Anak n=1, RTX n=5)			9 (13)	
Methotrexate, no (%)	41 (82)		29 (43)	
Methotrexate dosage, median (range) mg/week	15 (5-30)		NA	
Other DMARDs (HChl n=6, Sul n=2, Lef n=6, Aur n=1)			17 (20)	
no DMARDs			9 (10)	
Corticosteroids, no (%)	34 (68)		23 (34)	
Prednisone dosage, median (range) mg/dag	10 (5-15)		NA	

[#] Paired samples of synovial fluid and serum were analyzed from 12 patients.

* Average (±SD) age, disease duration, ESR and CRP of these 12 patients were 57.2 (9.4), 16.2 (15.9), 29.8 (24.6) and 17.8 (8.8), respectively.

ACPA, anti-citrullinated protein antibody; aTNF, anti-TNF; Anak, anakinra; Aur, auromyose; CRP, C reactive protein; DAS28, disease activity score based on 28 joints; DMARDs, disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; HChl, hydroxychloroquine; Lef, leflunomide; NA, not available. OA, osteoarthritis; RA, rheumatoid arthritis; RF, rheumatoid factor; RTX, rituximab; Sal, salazosporine; Sul, sulphasalazine; TNF, tumour necrosis factor.

In addition, κ and λ FLC positive cells were detected within the synovial tissue of patients with RA. Double staining of FLC and CD20 revealed that, despite a few exceptions, B cells are not positive for FLC (figure 1B,D). We did not observe profound FLC expression and plasma cell infiltration in synovial tissue from clinically stable patients with OA. Staining of serial tissue sections demonstrated that FLC positive cells and CD138 positive plasma cells are present in similar regions (figure 1B-D).

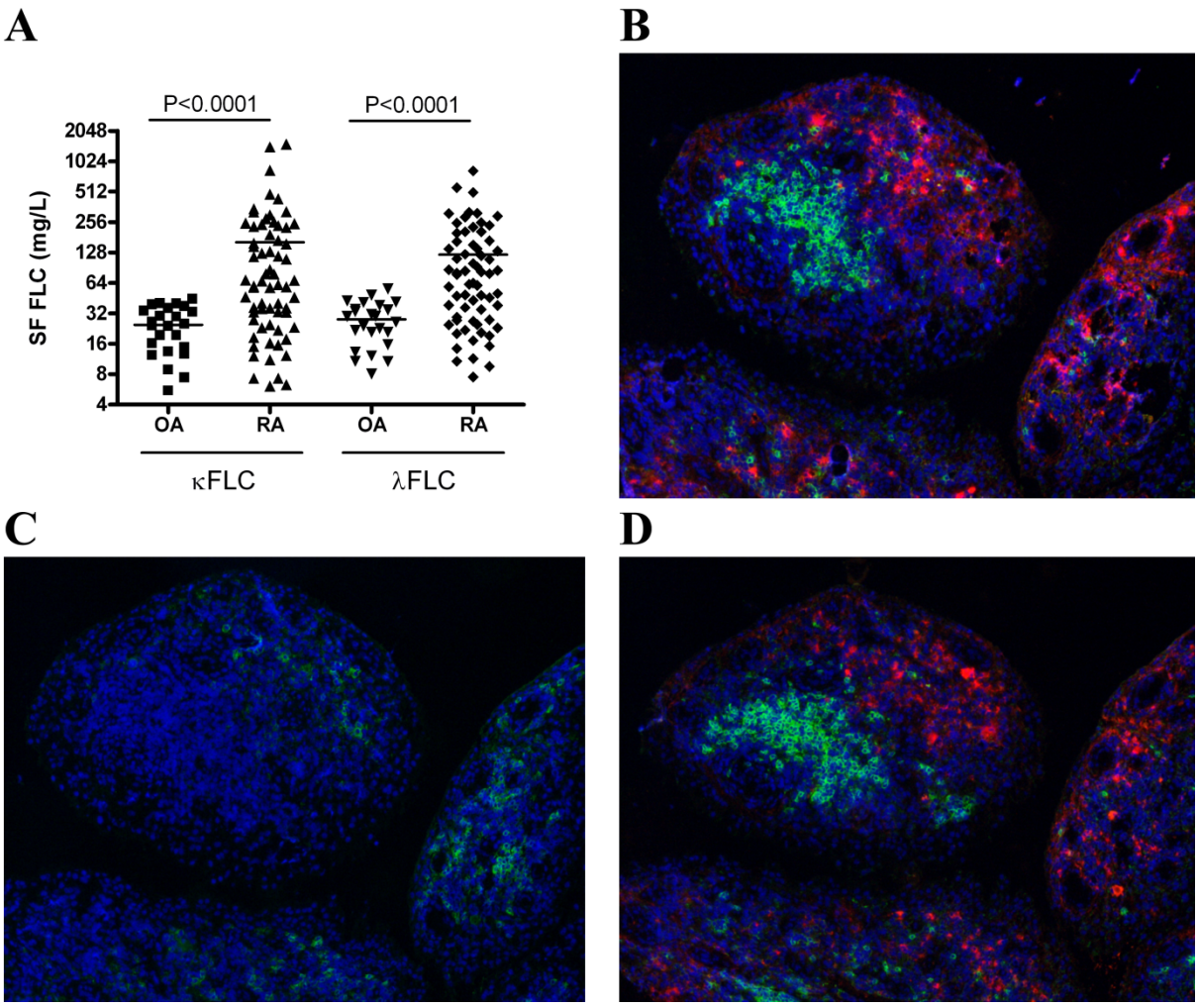

Figure 1. Immunoglobulin free light chains (FLCs) are abundantly present in the synovial fluid of patients with rheumatoid arthritis (n=67) compared to patients with osteoarthritis (n=24) **(A)**. Within the synovial tissue, κ and λ FLC positive cells were detected (**B** and **D**, respectively; red), which were not CD20 + B cells (**B** and **D**; green). Staining of sequential tissue sections indicated that a great number of FLC positive cells colocalize with plasma cells (**C**; green). K, kappa; λ, lambda; OA, osteoarthritis; RA, rheumatoid arthritis.

SF FLC concentrations correlate with serum FLC concentrations and inflammatory parameters

SF FLC concentrations highly correlated with serum FLC concentrations (κ and λ: r=0.98 and r=0.94, respectively, p<0.0001) (figure 2A,B). SF FLC concentrations were always

higher than serum FLC concentrations when serum FLC concentrations in patients with RA exceeded those of healthy controls (fig 2C,D), suggesting local production of FLCs. In addition, high local FLC concentrations correlated with high ESR (n=48, κ: r=0.58, P<0.0001 and λ: r= 0.52, p=0.0004) (figure 2E,F) and CRP levels (n=31, κ: r=0.63, p=0.0001 and λ: r=0.49, p=0.0048; data not shown).

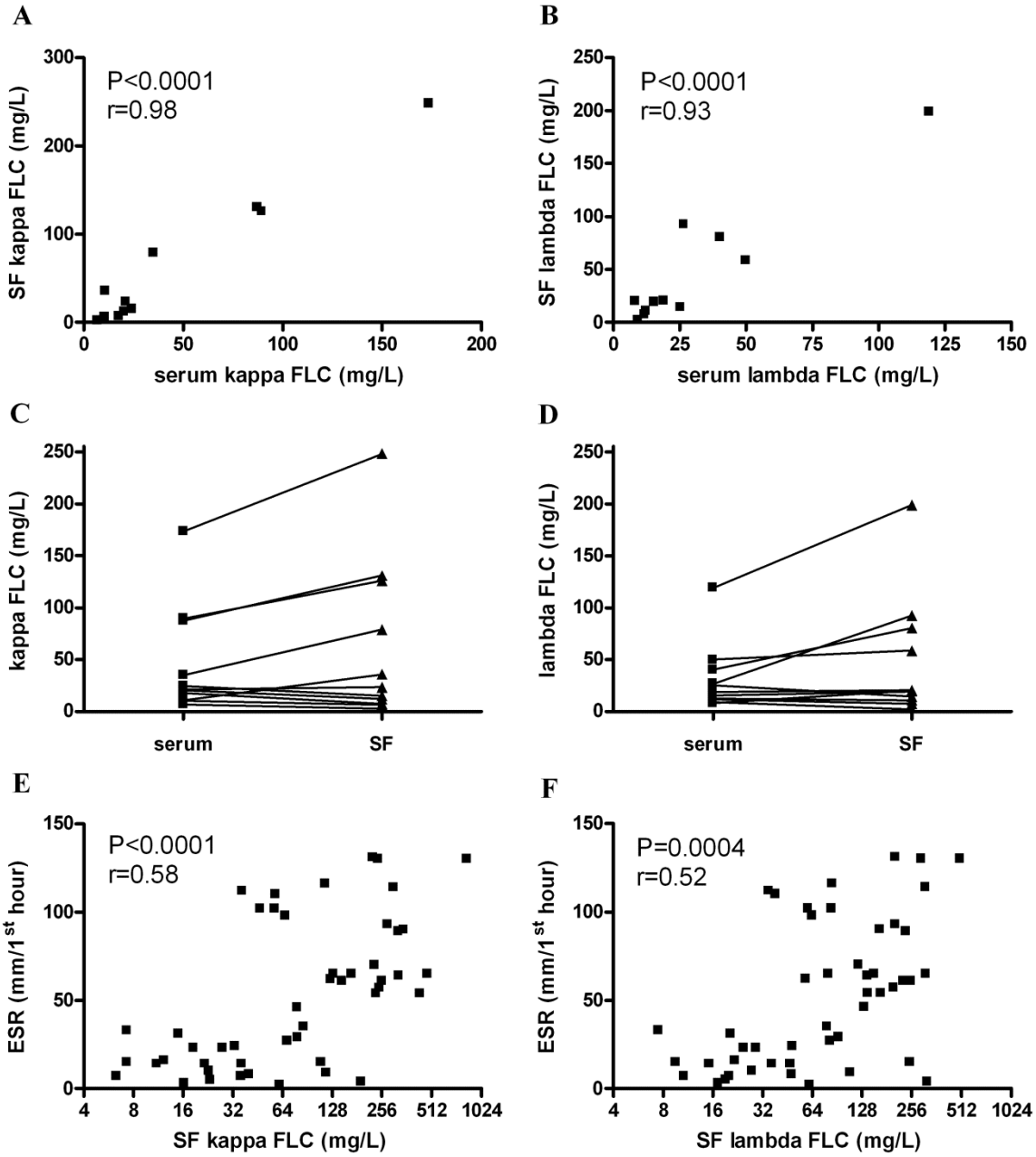

Figure 2. Local immunoglobulin free light chain (FLC) in synovial fluid (SF) highly correlates with systemic FLC concentrations for both kappa (**A**) and lambda (**B**) in patients with RA (n=11). Paired serum and SF kappa and lambda FLC concentrations of these individual patients are shown in (**C)** and (**D)**, respectively. SF kappa and lambda FLC both correlated with the systemic inflammatory parameter erythrocyte sedimentation rate (ESR; n=48) (**E** and **F**).

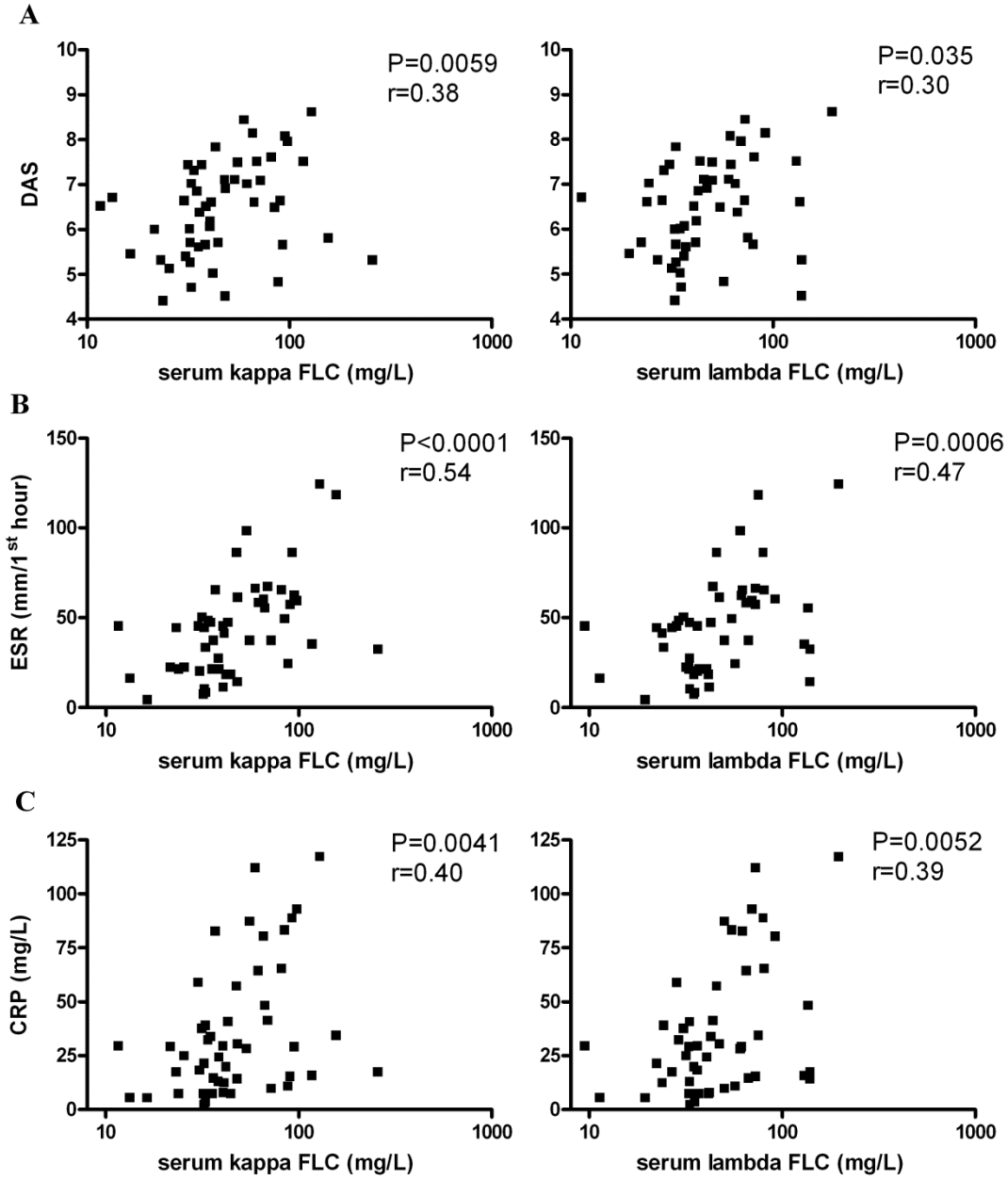

Figure 3. Kappa and lambda immunoglobulin free light chain (FLC) serum concentrations correlate with disease activity markers before the start of rituximab treatment. Kappa and lambda FLC concentrations both correlated significantly with (**A)**, Disease Activity Score (DAS), (**B)**, Erythrocyte Sedimentation Rate (ESR), and (**C)**, C Reactive Protein (CRP). p values and corresponding r values are shown in the graphs (n=50).

Relationship between serum FLCs and disease activity markers at baseline

At baseline, serum κ and λ FLC concentrations positively correlated with disease activity scores (DAS28; κ: r=0.38, p=0.0059 and λ: r=0.30, p=0.035). Similarly, FLCs significantly correlated with ESR (κ: r=0.54, p<0.0001 and λ: r=0.47, p=0.0006), and CRP (κ: r=0.40, p=0.0041 and λ: r=0.39, p=0.0052) (figure 3).

Decrease of serum FLC concentrations after rituximab treatment correlates with disease reduction

At baseline, mean serum κ and λ FLC concentrations were 56.3±6.17 and 53.5±5.31 mg/L (mean±SEM), respectively. κ and λ FLC concentrations significantly decreased as compared to baseline at 3 months (41.6±4.35 mg/L, p<0.001 and 42.7±4.33 mg/L, p<0.01, respectively), and 6 months (44.0±4.83 mg/L, p<0.001 and 43.0±4.03 mg/L, p<0.001, respectively) after rituximab treatment (figure 4A). In addition, FLC concentrations significantly decreased over time in patients that responded (good to moderate) according to EULAR response criteria (κ: -28.6±4.2% and -22.4±6.9%, p<0.0001, and λ: -23.9±3.1% and -18.1±7.5%, p<0.0001, at 3 and 6 months, respectively). By contrast, FLC concentrations in non-responders were not significantly decreased (κ: -0.2±11.4% and +10.5±16.5%, P=0.72 and λ: +5.8±15.3% and +10.2±21.3%, P=0.91, at 3 and 6 months, respectively) (figure 4B,C).

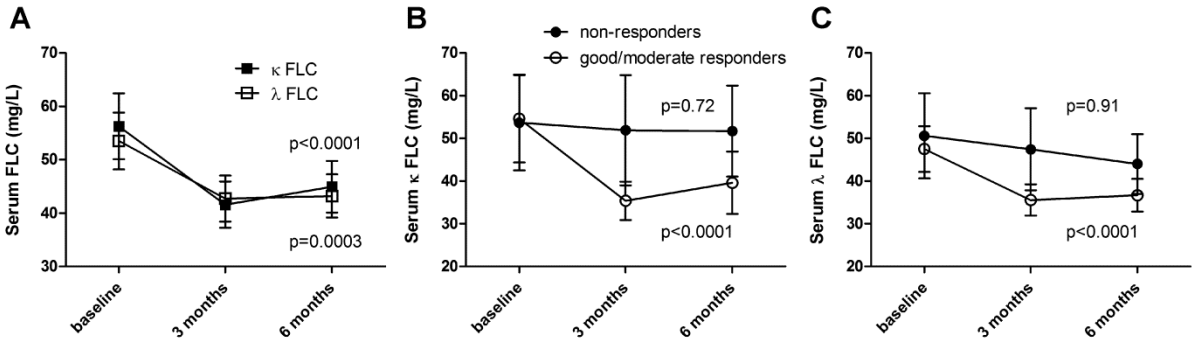

Figure 4. Changes in serum concentrations of κ and λ immunoglobulin free light chains (FLC) are associated with clinical response to rituximab. **A.** Total FLC concentrations significantly declined over time after rituximab treatment compared to baseline. Values are mean±SEM. When comparing changes in FLC concentrations at 3 and 6 months versus baseline in good to moderate (n=23) and non-responders (n=13) independently, a significant decrease in both κ **(B)** and λ **(C)** FLC concentrations was only found in the good to moderate responding group. Values are mean±SEM. κ = kappa; λ = lambda.

Besides the association between clinical efficacy based on the EULAR response criteria and changes in FLC concentrations, a decrease in serum FLC concentrations also correlated with a decrease in ESR at 3 months after start of treatment (κ: r=0.47, p=0.0006 and λ: r=0.40, p=0.0045) (figure 5A,C), as well as 6 months after start of treatment (κ: r=0.54, p<0.0001 and λ: r=0.45, p=0.0012) (figure 5B,D). Comparable correlations were found between changes in FLC and changes in CRP (κ: p=0.0047, r=0.40 and λ: p=0.027, r=0.32 at 6 months after treatment).

Different dosing of prednisone and/or methotrexate was not associated with significant changes in FLC concentrations (data not shown). Furthermore, baseline serum FLC

concentrations did not predict the degree of change in FLC concentrations, nor clinical outcome after rituximab treatment.

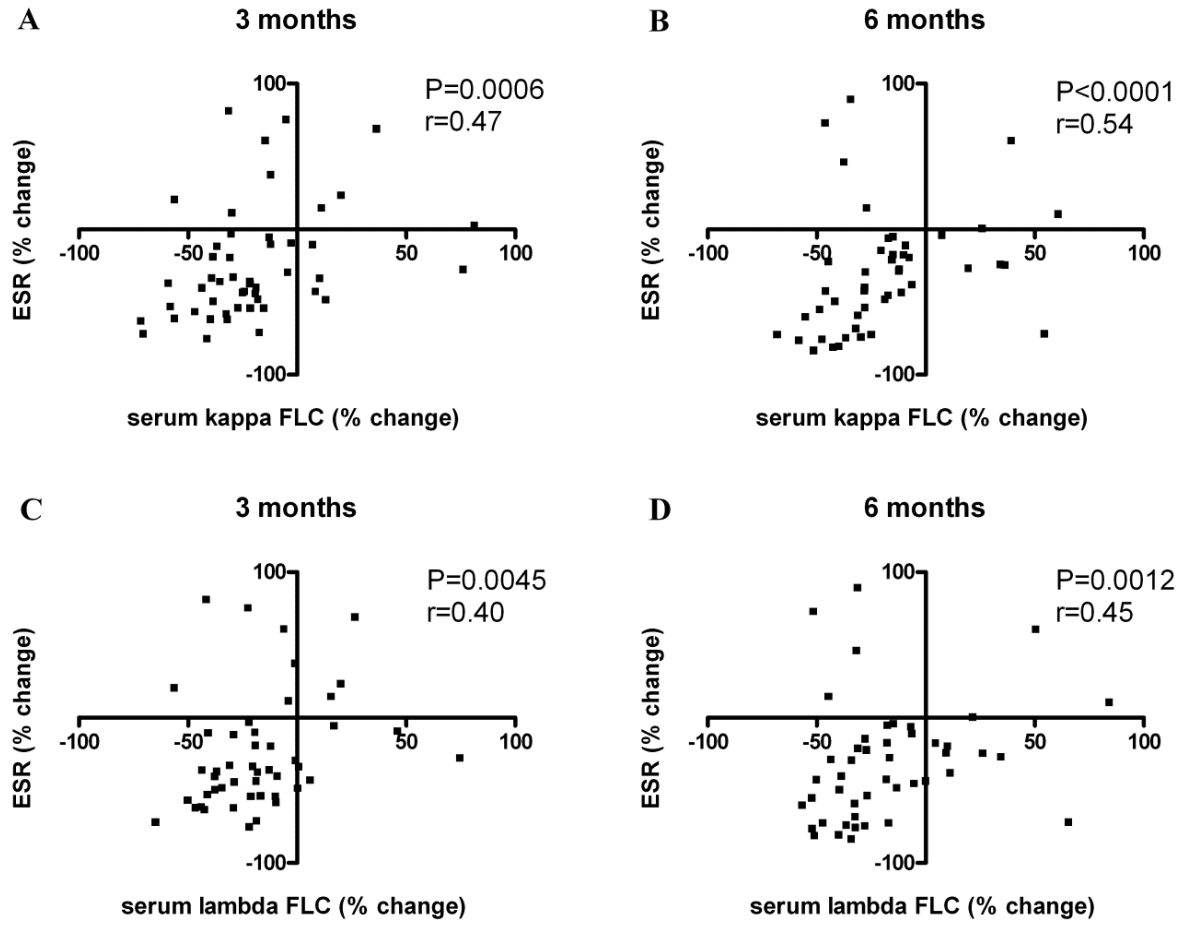

Figure 5. Changes in kappa and lambda immunoglobulin free light chain (FLC) concentrations correlate with changes inflammatory markers upon rituximab treatment. At both 3 and 6 months after the start of treatment, a decrease in kappa (**A** and **B**, respectively) and lambda (**C** and **D**, respectively) FLC concentrations correlated significantly with a decrease in Erythrocyte Sedimentation Rate (ESR) (n=50). p values and corresponding r values are shown in the graphs.

Effects of rituximab treatment on total Ig levels, IgG-ACPA and IgM-RF

Alterations in serum IgM, IgG and IgA concentrations were analyzed and compared to changes seen for serum FLCs. The decrease of total IgM after treatment was comparable to FLC, 18.1±3.2% and 19.5±2.7%, 3 and 6 months after treatment, respectively. In contrast to FLCs however, total IgM clearly decreased in good to moderate and non-responder patients ($p<0.0001$ and $p=0.017$, respectively) (see supplementary material, figure 6A [page 120]). Total concentrations of IgG and IgA only slightly changed after 3 (1.95±2.5% and 0.83±2.0%) and 6 months (2.73±2.3% and 3.79±2.1%), and remained within their normal range.

Concentrations of the antigen-specific arthritis-related antibodies IgG-ACPA and IgM-RF, were significantly different from baseline after treatment (both p<0.0001) (see supplementary material, figure 6B [page 120]). IgG-ACPA concentrations significantly decreased over time in good-to-moderate responders (p=0.0001) not in non-responders (p=0.66). IgM-RF concentrations also significantly decreased over time in good to moderate responders (p<0.0001) and in non-responders (p=0.016) (see supplementary material, figure 6C,D). Because patient data were only analyzed when all data from three timepoints were available, the number of non-responding patients became limited due to missing data at single timepoints. If all non-responding patient sera at 6 months after treatment were included, IgG-ACPA also significantly decreased compared to baseline values in non-responders (IgG-ACPA: p=0.035, n=15).

Discussion

In this study, we show that patients with RA have high concentrations of FLC in SF of affected joints and abundant expression of FLCs in the synovial tissue. Furthermore, increased serum FLC concentrations significantly correlated with SF FLC concentrations, disease activity, and markers of inflammation (ESR and CRP). B cell depletion by rituximab treatment resulted in decreased serum FLC concentrations only in patients with RA that respond to therapy.

FLCs are produced and secreted by B cells, plasmablasts and/or plasma cells. We found considerable numbers of FLC-positive cells present in synovial tissue of patients with RA. A substantial proportion of these cells colocalized with CD138+ plasma cells, but not CD20+ cells. Recently, we found comparable colocalization in mucosal tissue from patients with rhinitis.[16] The number of FLC-positive cells appears to exceed the number of CD138+ cells, suggesting that other cells in addition to plasma cells are positive for FLC. Additional to the substantial number of FLC-positive cells in RA synovial tissue, FLC concentrations were greatly increased in SF of patients with RA as compared to patients with OA, in which we did not observe profound FLC expression and synovial plasma cell infiltration. Moreover, serum FLC concentrations highly correlated with SF FLC concentrations. Together, our data indicate that FLCs are abundantly produced within the inflamed synovium of patients with RA, and that changes in serum FLC concentrations reflect changes in local FLC concentrations.

Serum FLC concentrations in patients with RA were also greatly increased compared to healthy controls. The clear correlation of both FLC subtypes and DAS28 and CRP values are consistent with previous findings which also showed increased concentrations of serum

FLCs in patients with RA which correlated with severity of disease and CRP.[14 18 27] In addition to stronger correlations between both κ and λ FLCs and CRP, in this study we find clear correlations between both FLC subtypes and ESR.

Ritixumab therapy eliminates peripheral CD20-positive B lymphocytes (mature and pre B cells) without dramatic effects on plasma cells. Peripheral B cell depletion in patients with RA significantly decreased total serum FLC concentrations. Interestingly, only good-to-moderate responding patients showed a significant decrease of both κ and λ FLC serum concentrations at 3 and 6 months after initiation of rituximab treatment compared to baseline. Serum FLC concentrations in the good-to-moderate responders did not always return to values found in healthy subjects, which may be explained by a continued local production in the synovial tissues. Synovial B cells and plasma cells are found in a substantial number of patients with RA, and the reduction of CD20+ B cells after rituximab treatment in tissue is much more variable compared to the (nearly complete) depletion in peripheral blood.[23 28] Moreover, even multiple cycles of anti-CD20 treatment does not return patients to an immune state in which all past cellular remnants of memory and autoimmune responses have been erased.[29]

Interestingly, in contrast to the effects of rituximab treatment on serum FLC concentrations, no significant changes were found in serum FLC concentrations 6 and 12 weeks after anti-TNF treatment (n=20, data not shown). This suggests that the changes seen in FLC concentrations after rituximab treatment may be part of the therapeutic action of rituximab and are not a general feature of clinical response to disease treatment.

Since plasma cells represent end-differentiated B-lineage cells and seem to be the predominant producers of FLCs, B cell depletion is likely to affect plasma cell numbers in the long term and thereby FLC production. Synovial plasma cell numbers do not differ between clinical responders and non-responders at baseline. However, a direct relationship between the decrease in synovial plasma cells and clinical improvement over time after rituximab treatment is reported.[28] Therefore, indirect depletion of synovial plasma cells may be an important mechanism by which rituximab decreases FLC concentrations. This may also include depletion of a proportion of CD38+CD138- plasmablasts, which are short-lived plasma cell precursors. Interestingly, changes in FLCs do not appear to reflect changes in total IgG and IgA, nor autoantibody concentrations. Which cell type is the major producer of FLCs and how FLC expression is regulated at a molecular level is unknown at this moment. However, our data suggest that possibly different types of plasma cells produce complete (auto)antibodies and FLCs.

Parallel to the divert FLC responses in clinical responders versus non-responders, we found clear correlations between changes in FLC concentrations and both ESR and CRP. Together this indicates that the percentage change in total κ and λ FLC serum concentrations may be an interesting measurement for the clinical response to rituximab therapy. In contrast, changes in RF and ACPA do not, or to a lesser extent, correlate with response to therapy, even though RF and ACPA are good prognostic biomarkers for RA development and progression at baseline.[12 18 30 31] Our data suggest that other B cell derived factors may be involved in RA pathology and changes in FLC may reflect changes in disease activity and treatment efficacy.

Future experiments have to reveal whether FLCs with antigen specificity for synovial tissue components, such as citrullinated proteins, the Fc portion of IgG, collagen type II and proteoglycans can be identified. This can be of significant importance since we have shown previously that FLCs can mediate antigen-specific mast cell activation.[32] Mast cells are suggested to play an essential role in RA pathology. Increased numbers are detected in the synovial tissue and mast cell mediators can be found in SF of patients with RA. Moreover, associations were found with proinflammatory cytokines and chondrolytic enzymes at sites of cartilage erosion.[33-35] It is tempting to speculate that FLCs could contribute to inflammation and immunopathology by triggering antigen-specific mast cell activation in affected joints.

In conclusion, we show that increased serum FLC concentrations observed in patients with RA correlate with increased SF FLC concentrations, disease activity, and inflammatory parameters. Besides, clinical response to rituximab treatment is accompanied with a significant reduction in serum FLC concentrations. Based on these data, we propose that changes in serum FLC concentrations, to a greater extent than IgM-RF and IgG-ACPA, could serve as a biomarker for the clinical response to rituximab therapy in RA. Further studies are needed to fully explore the role of (antigen-specific) FLCs in the pathogenesis of RA and its potential as therapeutic target in the treatment of disease.

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Supplementary material

Methods

Kappa and lambda FLC analysis.

Total serum or synovial fluid FLC concentrations were determined using an ELISA adapted from Abe *et al.*[1 2] In short, plates were coated with goat-anti mouse IgG (M4280, Sigma) and incubated o/n (4^{o}C). Subsequently, plates were blocked for 1 hour (RT) and incubated with mouse-anti human kappa or lambda Ig-FLC MAb's (obtained from Dr. A. Solomon, Tennessee). After incubation with different dilutions of samples and standards (The BindingSite), plates were incubated with HRP-labeled goat F(ab')$_2$-anti human kappa or lambda Ig light chain Ab's (AHI1804 and AHI1904, respectively, Biosource, USA). Finally, TMB was used as a substrate and the enzymatic reaction was terminated by adding 0.9 M H_2SO_4. Between incubation steps, wells were washed three times with PBS-T (0.1% tween-20). Per sample, at least three data points within the linear portion of the standard curves were used to estimate the FLC concentration.

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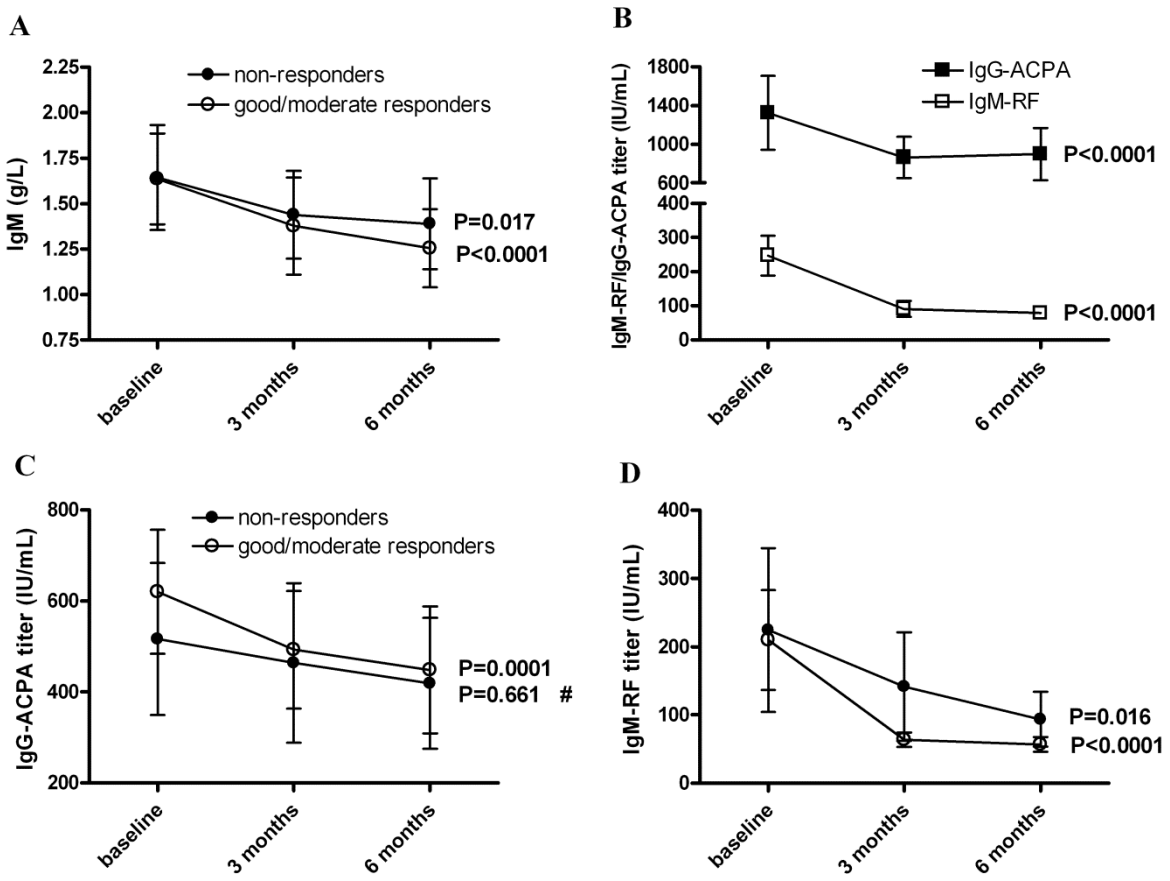
Figures



Supplementary figure 6. Rituximab treatment clearly declined total IgM concentrations in both good to moderate responder (n=14) and non-responder patients (n=10) **(A)**. When analyzing all treated patients, IgG-ACPA (n=40) and IgM-RF (n=33) concentrations also significantly decreased **(B)**. This decrease was significant in good to moderate responding patients for both factors (IgG-ACPA; n=20, IgM-RF; n=18) and in non-responders for IgM-RF when analyzing all timepoints (non-responders: IgG-ACPA; n=10, IgM-RF; n=8) (**C** and **D**, respectively). #, If only concentrations at baseline and 6 months after treatment were compared in all non-responder patient sera (n=15), decrease in IgG-ACPA also reached significance compared to baseline values (IgG-ACPA: P=0.04). Values are mean ± SEM.

Letter to the editor:

B cell activation biomarkers as predictive factors for the response to rituximab in rheumatoid arthritis: comment on the article by Sellam et al

Arthritis and Rheumatism, DOI 10.1002/art.30632

Janneke Tekstra, MD, Joel van Roon, PhD, Tom Groot Kormelink, MSc, Frank Redegeld, PhD

Sellam et al [1] examined whether serum B cell markers can predict the response to ritixumab in patients with refractory rheumatoid arthritis (RA). In a cohort of 208 patients, serum markers of B cell activation such as anti-cyclic citrullinated peptide (anti-CCP) antibodies, rheumatoid factor (RF), serum immunoglobulins (Igs), B cell activating factor (BAFF) and kappa and lambda Ig free light chains were measured at baseline. 149 of the patients (72 %) showed a good (21%) or partial (50%) response to the treatment according to the EULAR criteria [2]. Based on multivariate analysis, the authors conclude that presence of RF or anti-CCP antibodies *and* increased serum IgG predicts the response to rituximab treatment in patients with refractory RA.

However, the effect of rituximab on these biomarkers during the treatment period has not been measured nor discussed in this study. With respect to this, in recent work we have shown that rituximab therapy reduces serum concentrations of Ig free light chains [3]. In this study, serum of 50 RA patients was analyzed at baseline, and 3 and 6 months after rituximab treatment. We found that only patients with a good and moderate response to rituximab showed significant decreases in serum kappa and lambda Ig free light chains, while anti-CCP antibodies and RF were not significantly related to efficacy of treatment. Decreases in free light chain concentrations in rituximab responders correlated well with decreases in DAS28 [4], erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) and the changes in free light chains were not a reflection of decreases in serum IgG or IgA. The fact that regulation of free light chains, in contrast to complete antibodies, correlates with efficacy of treatment may be related to the difference in half-lives of these molecules. Change in B cell activity may thus better be monitored by measuring free light chains than by measuring IgG antibodies. In accordance with the study by Sellam et al, serum levels of Ig free light chains before the start of treatment were not found to be prognostic for efficacy of the rituximab treatment.

Serum Ig free light chains have been shown to be increased in several inflammatory diseases and correlate well with clinical parameters for disease activity markers in RA such as DAS28, ESR and CRP [3, 5-7]. Furthermore, serum free light chain concentrations highly correlate with local Ig free light chains concentrations in synovial fluid, which have also been found to be greatly increased in the inflamed joints of patients with RA, but not in osteoarthritic joints [3]. Interestingly, normalization of serum Ig free light chain levels in patients with systemic lupus erythematosus after rituximab treatment also correlated with a decrease in disease activity [8].

Taken together, these observations suggest that measurement of B cell activation markers in serum may be useful not only to predict, but also to monitor, response to rituximab in patients with RA. However, present evidence indicates that different biomarkers should be investigated when they are being analyzed for predicting (i.e. RF, anti-CCP, total IgG), versus monitoring (i.e. Ig free light chains) rituximab response.

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Reply

Arthritis and Rheumatism, DOI 10.1002/art.30631

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We thank Dr. Tekstra and colleagues for their interest in our study demonstrating that the presence of autoantibodies and increased serum IgG levels independently predict the response to rituximab in rheumatoid arthritis. The objective of this work was not to monitor serum B cell biomarkers after rituximab administration, but to identify baseline predictive markers of response to the drug. Since there are now 9 biologic agents available for the management of A, the identification of predictive factors before treatment institution is particularly challenging with regard to the goal of practicing personalized medicine to appropriately tailor the treatment to the individual patient [1].

We addressed the question of baseline pretreatment serum free light chain levels as a possible predictive marker

of response to rituximab, but, consistent with the findings of Tekstra and colleagues' group, neither the absolute value of serum free light chains nor above-normal serum free light chain levels before rituximab treatment was associated with clinical response 24 weeks after rituximab administration [2].

However, we agree that serum free light chain levels in RA have been found by several groups, including ours, to be an accurate and easily assessed surrogate biomarker of disease activity [3,4]. Tekstra et al's demonstration that serum free light chain levels are associated with clinical response to rituximab [3] provides additional data in support of this. But it does not mean that the association is linked to the mechanism of action of rituximab and, as free light chains are correlated with disease activity, exactly the same finding might be observed with any effective treatment of RA.

This discussion clearly illustrates that, as we are reminded by Tekstra et al, it is important to differentiate absolute values of baseline pretreatment biomarkers that are truly predictive of efficacy of a treatment from changes in biomarker levels after treatment, which may or may

not be specific to the treatment and, more importantly, may not be more accurate than classic clinical indexes for determining response to a drug.

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7

Mast cells as target in cancer therapy

This chapter is published in *Current Pharmaceutical Design*, 2009;15:1868-1878

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Abstract

A close interaction of cancer cells with their microenvironment is important for their growth and survival. In this respect, the involvement of inflammatory cells in the initiation, promotion and progression of cancer has pointed to new therapeutic opportunity in the treatment of cancer. The main immune cell types implicated in tumor-associated inflammation are macrophages, dendritic cells, lymphocytes, neutrophils, eosinophils and mast cells. Their precise role in, intercellular communication, regulation of tumor inflammation, and to what respect this inflammation contributes to tumor development, are not completely understood. Mast cells are key effector cells in allergic diseases, but it has become apparent that they also contribute to other pathologies, including autoimmune diseases and cancer. Activated mast cells can release many pro-angiogenic and tumor growth stimulatory mediators. Increased numbers of mast cells are found in many tumors and it has been shown that the number of tumor infiltrating mast cells correlate with increased intratumoral microvessel density, enhanced tumor growth and tumor invasion, and poor clinical outcome. Therefore, modulating mast cell recruitment, viability, activity, or mediator release patterns at malignant sites can be of importance to control tumor growth. In this review, we will focus on the contribution of mast cells to tumor development and growth and the possibilities to interfere in mast cell activation and proliferation in the therapy of cancer.

1. Mast cells

Mast cells are generated from hematopoietic progenitor cells and are released from the bone marrow as immature cells. Mature mast cells cannot be recovered from the blood stream. Instead, the immature mast cells distribute throughout the body and mature in the tissues in which they will ultimately reside during their lifespan [1, 2]. Although mast cells are located in virtually every vascularized tissue, most mast cells are positioned close to blood vessels and nerves and at anatomical sites that are in direct contact with the environment, like the skin, airways and gastrointestinal tract [3, 4]. Since maturation takes place in different body parts under different microenvironmental conditions, immature mast cells evolve into phenotypically distinct mature populations. This results in a profound heterogeneity of mast cells throughout the body [1]. which becomes apparent by differences in protease and glycosaminoglycan contents of granules, as well as differences in lipid-mediator production [5]. In addition, mast cells can proliferate and show extensive phenotypic plasticity after appropriate stimulation with a variety of factors, acting through autocrine, paracrine and/or systemic mechanisms [1, 6]. Stem cell factor (SCF) is the major survival and developmental factor for mast cells. Together with interleukin-3 (IL-3) and T helper type 2- associated cytokines like IL-4 and IL-9, this is the most important factor that influences proliferation and phenotypical aspects of mast cells. Moreover, a wide array of other cytokines, growth factors and chemokines can affect mast cells [6, 7].

Mast cells are most commonly known as the main effector cells in allergic, IgE-mediated immune reactions. In sensitized hosts, crosslinking of cognate antigen to IgE molecules that are bound to their high affinity receptor (FcεRI) activates mast cells to release their mediators, resulting in an inflammatory response [2, 8]. This process, however, can also be beneficial to the host, since this mechanism is well known to be involved in resistance to parasites [2, 9, 10]. In addition, mast cells can be activated by many other immunological and non-immunological signals, such as IgG, immunoglobulin free light chains, complement factors, bacteria, viruses, cytokines, inflammatory mediators, toll like receptors, and by T cells [5, 6, 11-13]. Depending on the type and strength of the activating stimuli, mast cells can release distinct patterns of a large array of mediators [3]. Three main classes of mediators can be distinguished [3, 5, 14]:

- pre-formed, granule-associated mediators (e.g. histamine, tryptase and heparin), and
- *de novo* synthesized lipid mediators (e.g. LTC_4, LTB_4 and PGD_2) and
- a variety of cytokines and chemokines

Mediator release can be accomplished by complete degranulation or by so-called piecemeal degranulation, leading to a less selective or more selective release of mediators, respectively [12, 14]. In this way, mast cells can initiate or maintain an appropriate and effective immune response, dependent on the activating signals. Mast cells not only function as effector cells, they can also act as regulatory cells. Mast cell mediators can exert proinflammatory, anti-inflammatory and/or immunoregulatory effects by influencing development, survival, proliferation, migration, maturation or function of other immune cells, like dendritic cells, B cells, T cells, macrophages, neutrophils and eosinophils [6, 7, 15-20]. Because of these immunomodulatory functions, mast cells are believed to be involved in both innate and adaptive immune responses and, thus, protect the body against harmful antigens and pathogens. Moreover, mast cells can also exert immunosuppressive effects [21-23] and induce tolerance mediated by regulatory T cells [24].

Together, these data establish that the mast cell has an important function in orchestrating many immune responses. These immune responses are not always beneficial to the host, underlined by the critical involvement of mast cells in allergic disorders. However, in recent years, it has also become apparent that mast cells contribute to other pathologies, such as autoimmune diseases [25, 26] including rheumatoid arthritis [27-30] and multiple sclerosis [31-34] and cardiovascular diseases [35-37].

2. Cancer and inflammation; critical role for mast cells?

The transformation of normal cells into malignant cells by genetic and epigenetic changes has generally been considered to result from activation of oncogenes that enhance unlimited cell proliferation, promote escape from apoptosis, stimulate angiogenesis, and increase invasion of surrounding tissues. During the last years, it is becoming apparent that the tumor microenvironment is a critical determinant of malignant transformation and tumor proliferation [38]. The tumor microenvironment mainly consists of tumor cells, stromal fibroblasts, epithelial cells, cells forming blood and lymphatic vessels, inflammatory cells and extracellular matrix components.

The observation that tumors contain inflammatory cells has already been recognized back in 1863 by Virchow. This could either mean that the inflammatory cells are active in fighting the malignant cells, stimulate tumor progression or a mix of both processes. The immune system may have a protective role in tumor development, known as cancer immunosurveillance. This process seems especially effective in virus-induced cancers, where a compromised immune system sparks a sharp increase in cancer incidence (e.g. Kaposi sarcoma in HIV-patients). In general, however, immunosurveillance likely eliminates

a portion of early-stage transformed cells, whereas clinically advanced tumors probably avoid immunosurveillance by inducing immune tolerance [39, 40]. Recently, the involvement of inflammation in the initiation, promotion and progression of cancer has received more attention [38, 41-45].

Table 1. Increased mast cell numbers in human cancers and positive correlations between tumor-associated mast cell numbers and various clinicopathologic features.

Increased mast cell number	
Basal cell carcinoma [151-153]	Endometrial carcinoma [154]
Melanoma [155-158]	Laryngeal squamous cell carcinoma [159]
Pancreatic ductal adenocarcinoma [160]	Renal cell carcinoma [161]
Oral squamous cell carcinoma [162, 163]	Gastric cancer [164]
Lung cancer [165-167]	Multiple myeloma [168]
Breast carcinoma [169]	B-cell non Hodgkin's lymphoma [170]
Correlation between mast cell number and increased microvessel density/angiogenesis	
Melanoma [157, 171, 172]	Gastric cancer [164]
Pancreatic ductal adenocarcinoma [160]	Multiple myeloma [168]
Oral squamous cell carcinoma [162]	B-cell non Hodgkin's lymphoma [170]
Lung cancer [165-167, 173, 174]	Colorectal carcinoma [175]
Endometrial carcinoma [154]	Uterine cervix carcinoma [176]
Renal cell carcinoma [161]	Esophageal squamous cell carcinoma [177]
Correlation between mast cell number and increased invasive ability/metastasis	
Melanoma [172]	Gastric cancer [164]
Pancreatic ductal adenocarcinoma [160]	Colorectal carcinoma [175, 178]
Lung cancer [174]	Esophageal squamous cell carcinoma [177]
Correlation between mast cell number and tumor stage/poor prognosis	
Melanoma [157, 171]	Uterine cervix carcinoma [176]
Lung cancer [166, 174]	Esophageal squamous cell carcinoma [177]
Endometrial carcinoma [154]	Prostate cancer [179]
Gastric cancer [164]	Hodgkin's lymphoma [180]
Colorectal carcinoma [175, 178]	

The main immune cell types implicated in tumor-associated inflammation are macrophages, dendritic cells, lymphocytes, neutrophils, eosinophils and mast cells. Their precise role, communication, and regulation of tumor inflammation and how this inflammation contributes to tumor development are not completely understood. In this review, we will focus on the contribution of mast cells to tumor proliferation and possible strategies to interfere with mast cell activation.

2.1 Mast cell and tumor development

The mast cell appears to be an attractive target in tumor therapy. In humans, increased levels of mast cells are found in many tumors. Moreover, it has been shown that the number of tumor infiltrating mast cells correlates with increased intratumoral microvessel density, enhanced tumor growth and tumor invasion, and poor clinical outcome (Table 1). The observed correlations indicate an association between mast cells and tumor growth. This clinical observation, together with *in vitro* data and data from rodent tumor models, suggests an important role for mast cells in the development and progression of cancer [46-49]. An *in vitro* study using the human mast cell line LAD2, for example, showed that mast cells regulated migration of cervical cancer cells by the release of histamine and cannabinoids, suggesting that mast cells could contribute to cervical cancer cell invasion and spreading [50]. In addition, this study showed that factors secreted by cervical tumor cells stimulated LAD2 cell degranulation, demonstrating a cross-talk between tumor cells and mast cells (figure 1). Besides these in vitro data, there are a few studies using rodent tumor models in which a more clear-cut involvement is demonstrated of mast cells in tumor development. In 1988, it was already shown that mast cell-deficient mice exhibited a decreased rate of tumor angiogenesis compared to their wild-type littermates. In addition, development of spontaneous lung metastases was less frequent in mast cell-deficient mice. Reconstitution of the mast cell population largely restored the wild type phenotype [51]. This role of mast cells in angiogenesis is supported by studies in a HPV16 transgenic mouse model of squamous epithelial carcinoma [52]. Mast cell infiltration was found in hyperplasias, dysplasias, and invasive fronts of carcinomas together with the activation of metalloproteinase-9 (MMP-9). This coincides with the angiogenic switch in premalignant lesions. Degranulation of mast cells, releasing chymase and tryptase, was observed in close proximity to capillaries and epithelial basement membranes. The authors showed that tryptase is mitogenic for dermal fibroblasts that proliferate in reactive neoplastic stroma, and that chymase can activate MMP-9, which leads to extracellular matrix (ECM) remodeling. More important, mast cell deficiency resulted in a severe attenuation of early neoplasia, probably due to an impaired vascular response. Recently, a study by Soucek *et al*. [53] further supported the essential role of mast cells in tumor angiogenesis and growth. These authors used a transgenic Myc-induced pancreatic islet tumor model, in which they can reversibly switch Myc-expression. The Myc oncoprotein, a pleiotropic transcription factor that is overexpressed in many human cancers, rapidly induced the expression of several chemokines. This resulted in the predominant recruitment of mast cells within the first 24 hours. Macrophages and neutrophils were only found after one week of sustained Myc activity, occurring after onset of tumor angiogenesis. Subsequently, they demonstrated that

mast cells were essential for tumor growth, since blocking mast cell degranulation using cromolyn, completely blocked Myc-induced islet expansion. In mast cell-deficient mice (W-*Sash*), the exact same phenotype as in wild-type mice treated with cromolyn was observed. Furthermore, the observed tumor growth inhibition appeared due to a deficit in the maintenance of tumor angiogenesis. In this model, functional mast cells seem required to maintain established tumors.

These studies put forward a critical role for mast cells in tumor angiogenesis. Angiogenesis is crucial for macroscopic expansion of human tumors and essential for metastasis [54, 55]. This role for mast cells is not surprising given the capacity of these cells to produce and release a large array of potent angiogenic cytokines and proteases. As reviewed by Ribatti *et al.*, mediators like VEGF, FGF-2, TGF-β, TNF-α, IL-8, NGF, tryptase, chymase, heparin, and histamine can be involved in mast cell-induced angiogenesis [56]. Moreover, MMP-2 and MMP-9 can be synthesized and secreted by mast cells [57, 58], or activated by mast cell mediators [52, 59, 60]. This causes ECM remodeling, which releases angiogenic factors that are sequestered in the ECM (such as VEGF, FGF and TNF-α) that further intensify tumor-associated angiogenesis [44] and may facilitate invasion [61].

Next to the involvement in angiogenesis and the breakdown of surrounding stroma, mast cells also appear to be involved in promotion of tumor cell proliferation, and inhibition of the immune response against neoplastic cells. The mitogenic effect of the mast cell mediators FGF-2 and IL-8 is shown on melanoma cells [62, 63]. IL-6 enhances cancer cell proliferation in multiple myeloma [64, 65] and the same accounts for tryptase in a colon carcinoma cell line [66] and in a model for cheek pouch carcinoma [67]. Moreover, histamine and growth factors such as SCF, NGF, and PDGF can induce tumor cell proliferation [49]. The immunosuppressive effects of mast cells are supported by a recent finding that SCF-activated mast cells exacerbate tumor immunosuppression in mice by releasing adenosine and increasing regulatory T cells. This suppressed T cells and NK cells within the tumor [68].

Despite this substantial amount of evidence for the role of mast cells in different aspects of tumorigenesis and tumor proliferation, the exact mechanisms leading to the recruitment and activation of mast cells and other inflammatory cell types, are still undefined. Some factors are likely candidates for attracting mast cells to neoplastic lesions and for their activation. Of these, SCF is probably the most important. SCF is a powerful mast cell chemo-attractant and activator [69, 70]. Increased production of SCF is found in several human tumor cell lines [71]. When SCF-expression was boosted by transfection of tumor cells with SCF-encoding DNA, the number of infiltrating mast cells in the tumor increased and enhanced tumor vascularization compared to control tumors was observed. This was in contrast to

tumor cells transfected with non-coding DNA, where tumors showed lower mast cell numbers, decreased vascular density, and diminished tumor growth [72]. Recently, Huang *et al.* also demonstrated SCF expression in human and murine tumor cell lines and corresponding tumor tissues. The authors showed the potential of SCF to recruit mast cells to (low SCF concentration), and activate mast cells (high SCF concentration) within tumor tissue [68]. These tumor-infiltrating mast cells were found to produce many inflammatory factors, to increase IL-17 expression in tumor cells, and to regulate immunosuppression. This suggests that mast cells do not only participate in remodeling the tumor microenvironment, but also critically regulate inflammation and immunosuppression.

The expression of adrenomedullin can be upregulated by hypoxia [73, 74]. This peptide is abundantly produced in a variety of cancers [75]. Adrenomedullin is chemotactic for human mast cells and it can induce histamine and β-hexosaminidase release by mast cells, and stimulate expression of VEGF, MCP-1, and FGF-2 [76]. This might indicate that hypoxia serves as an initial signal leading to mast cell recruitment. Besides SCF and adrenomedullin, other mast cell chemoattractants like NGF, RANTES, MCP-1, VEGF, bFGF, PDGF and activators, including NGF, IL-1, and CRH have been identified [12, 77]. Putatively, these factors are involved in mast cell recruitment and/or activation in cancer as well. For MCP-1 this is suggested by the study of Soucek *et al.*, in which Myc activation resulted in a high and rapid induction of MCP-1 expression [53].

So far we have discussed mediators that promote tumorigenesis and tumor growth. Nevertheless, mast cells also have the ability to synthesize and release mediators which can be harmful to the tumor. A tumor suppressive action has been described for TGF-β [78], IL-10 [79], and IL-4 [80], whereas a direct cytotoxic effect has been shown for TNF-α [81]. As summarized by Galinsky *et al.*, mast cell heparin can also reduce tumor growth [46]. Other mast cell mediators that have been described to inhibit cancer are IL-1, IL-6, IFN-α, and arachidonic acid products [49]. These findings underline the seemingly opposite roles of mast cells in cancer. They are further supported by clinical observations that show a positive correlation between mast cell number and prognosis in several tumor types, especially in breast cancer [82-84], colorectal cancer [85, 86], and non-small cell lung cancer (NSCLC) [87, 88]. Theoharides *et al.* proposed that this dual role of mast cells can be explained by the selective release of mediators, regulated by distinct signals [49]. Thus, depending on specific microenvironmental conditions, mast cells might be exposed to signals that either promote or inhibit tumorigenesis. In turn, the tumor microenvironment possibly influences the ultimate response by different mediators, since some mediators can exert both pro- and anti-tumor effects (figure 1).

Modulating mast cell recruitment, viability, activity, or mediator releasing patterns at malignant sites can be of importance to control tumor growth. In the following paragraphs, we will discuss important pathways that are active in mast cells that could be targets for pharmaceutical intervention.

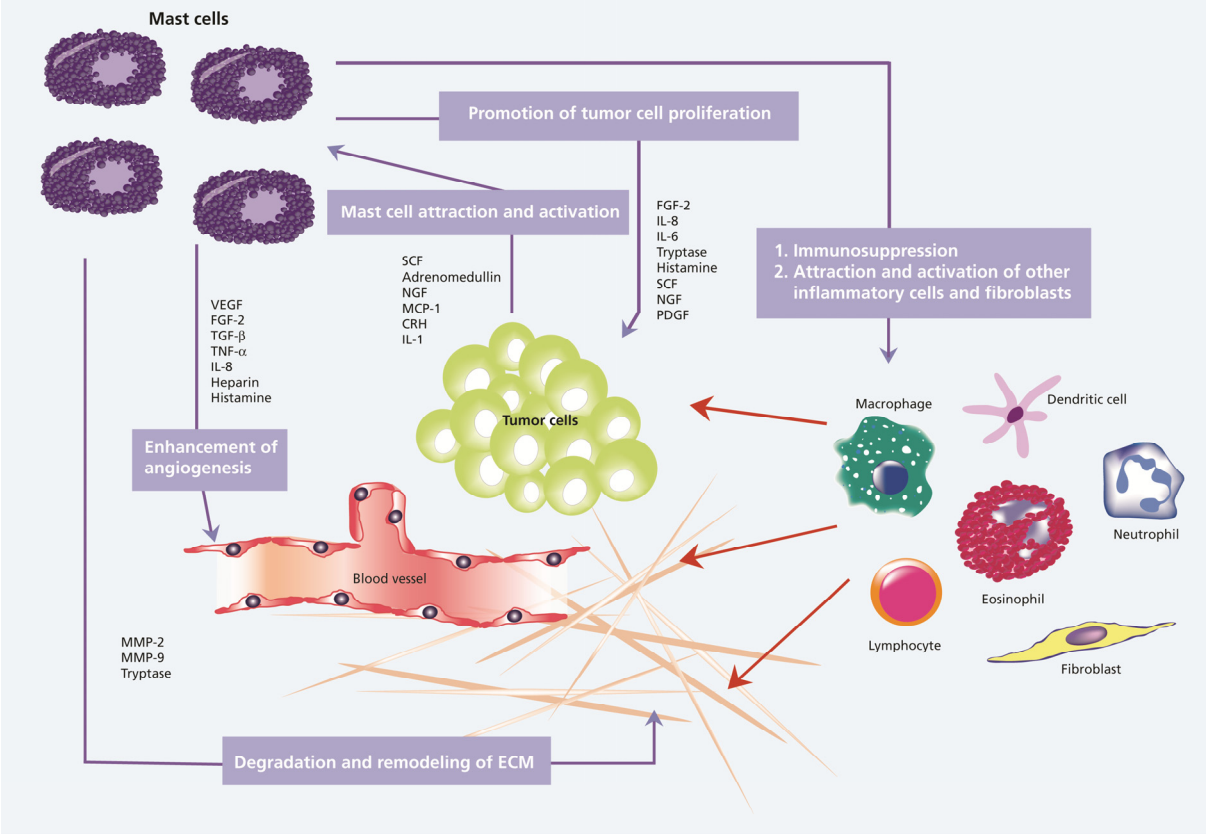

Figure 1. Possible contribution of tumor-associated mast cells to tumor progression. During neoplastic progression, various cancer tissues release chemotactic factors which recruit mast cells to the tumor microenvironment. There, mast cells become activated to (selectively) secrete a plethora of mediators. This results in a pro-tumor microenvironment by the enhancement of angiogenesis, promotion of tumor cell proliferation, degradation and remodeling of the extracellular matrix (ECM), and immunosuppression. In addition, mast cells are possibly involved in the recruitment and activation of other inflammatory cells and fibroblasts, often found in tumor stroma. On their turn, these cells can further augment tumorigenesis by enhancing tumor cell proliferation, angiogenesis, and extracellular matrix remodeling.

3. Mast cells as possible target for cancer therapy

The signaling pathways that regulate mast cell degranulation have been studied extensively, especially in allergy research. As discussed above, mast cells generate and release several kinds of mediators. In the next sections, we will discuss some approaches to inhibit activation and proliferation of mast cells or to neutralize its released mediators.

3.1 Mast cell stabilizers

Mast cell activation has two distinct phases, the first consisting of release of preformed mediators occurring within minutes and the second consisting of release of newly synthesized molecules at 3-12 hours later. Cromolyn, the archetype mast cell stabilizer, appears to be effective against both reactions. Currently there are over 20 compounds identified that inhibit mast cell activation, but may also have additional features. Olopatadine demonstrates mast cell stabilizing activity, but also acts as an anti-histaminic [89]. Ketotifen is a mast cell stabilizer used to treat asthma which can also help to prevent fibrosis [90]. Iodoxamide tromethamine is a mast cell stabilizer that inhibits in vivo type I immediate hypersensitivity reactions and inflammation [89].

Treatment of breast carcinoma with cimetine, a H2 receptor antagonist, showed that it may influence histamine activity or mast cell activation [91]. JNJ 7777120 is a new H4 receptor antagonist and H4 receptor has been identified on human mast cells. H4 receptor activation induces calcium mobilization in mast cells but does not affect IgE crosslinking-induced degranulation [92]. Drugs as azelastine and episastine have been examined to inhibit IgE-mediated phosphorylation of FcεRIγ protein [93]. Many other compounds such as syk kinase inhibitors [94, 95], MAP kinase inhibitors, ion channel antagonists [96, 97] and sphingosine kinase inhibitors [98] could be used to block mast cell activation, but in most cases also activation of other cells may be influenced.

3.2 Protease inhibitors

Tryptase is considered to be a pro-inflammatory mediator and increased tryptase levels reflect ongoing mast degranulation. Increased concentrations of tryptase in biological fluids can be found in several disorders, including systemic anaphylaxis, and other hypersensitivity and autoimmune disorders [99, 100]. In addition, as discussed above, mast cells act at sites of new vessel formation in part by secreting tryptase which then functions as a potent angiogenic factor [101, 102]. Mast cell tryptase can be targeted by various tryptase inhibitors and notable compounds include guanidino dipeptide, guanidino dimers, delta inhibitors, benzamidine dimers etc, most of them have a preclinical application [100].

3.3 IgE and anti-IgE in the treatment of cancer

Treatment with anti-IgE (Omaluzimab; Xolair^{TM}) reduces the free IgE concentrations in the circulation. Interaction of IgE with its high affinity receptor FcεRI without the need of receptor crosslinking by antigen has been shown to increase the survival of mast cells. Therefore, in theory, anti-IgE treatment may reduce the number of mast cells in inflammatory conditions.

However, it was shown in a recent study that treatment of patients with anti-IgE (Xolair™) reduces the number of basophils in the circulation, but not mast cells in the skin [103]. This lack of effect may be due to the long half life of skin mast cells. Thus far, anti-IgE treatment has not been applied in cancer patients.

On the other hand, specific IgE could be used to specifically target tumor cells and to activate tumoricidic effector cells such as eosinophils, mast cells and macrophages (for review see [104]).

3.4 Stem Cell Factor (SCF) and c-kit mediated signaling pathway

Mast cell degranulation can be influenced by stem cell factor (SCF) and its cognate receptor c-Kit. Therefore, pharmaceuticals interfering with SCF/kit or their downstream signals may alter mediator release (patterns) by mast cells.

3.4.1 SCF

SCF, also known as c-Kit ligand, is a glycoprotein which is the major survival and developmental factor for mast cells as described above. SCF can be expressed in two active isoforms [105]. One isoform is a 248 amino acids long protein, known as sSCF248. It is membrane bound in the full length form, but it contains a specific proteolytic cleavage site at Ala165 (Val-Ala-Ala-Ser, aa 163-166), which generates a soluble form of 164 amino acids length. The second isoform is 220 amino acids long and is membrane-bound (mSCF220). mSCF220 lacks the proteolytic cleavage site and remains a transmembrane protein that consists of a 157 amino acid extracellular domain, a 27 amino acid transmembrane domain and a 36 amino acid cytoplasmic tail (reviewed in [106]). The ratios of the two isoforms vary considerably in different cells and tissues. In vitro experiments provide evidence for differential functions of mSCF and sSCF. For example, the phenotype of Sl/Sld mice which produce only sSCF due to a deletion of the transmembrane and cytoplasmic domain, provides strong evidence for an essential role of mSCF and a less important role of sSCF, as these mice are mast-cell deficient due to lack of the correct micro-environmental signals for mast cell development [107].

SCF is vital for mast cell survival, degranulation and release of inflammatory-cytokines. The presence of SCF amplifies the antigen-stimulation signal leading to pro-inflammatory/pro-angiogenic mediator release from mast cells. This is the result of the interaction of SCF with c-Kit to activate calcium mobilization, which is an important step in the degranulation. SCF, by itself, can also stimulate mast cell degranulation irrespective of antigen stimulation [108].

At the molecular level, SCF is generated in a variety of cell types in response to diverse intracellular signals. For example, IL-18 enhances SCF-production in B16 murine melanoma cells through pathways involving ROI and activation of p38 MAP kinase [109]. SCF production is also increased by IL-1β, TNF-α or phorbol 12-myristate 13-acetate (PMA) in vascular endothelial cells [110] and lung fibroblasts [111].

As discussed above, SCF is expressed by several human and murine tumor cell lines and corresponding tumor tissues [68], indicating that tumor cells may stimulate mast cell degranulation and that SCF inhibition in tumors may be beneficial. In a clinical setting, SCF is primarily known for its ability to stimulate unwanted histamine production in allergic reactions. The treatment is generally aimed at inhibiting these symptoms and usually consists of prophylactic antihistamines, an H2-receptor antagonist and β-adrenoreceptor stimulant inhaler [112]. Glucocorticoids interfere with the SCF pathway, as the drugs can downregulate SCF production as demonstrated in lung fibroblasts, thereby reducing levels of the protein [111]. Alternatively, SCF could be targeted directly. There are two methods to control the level of SCF in a direct manner. One is the use of neutralizing antibodies to reduce the availability of SCF and bring the concentration to human physiological levels, which averages 3.3 ng/mL [113]. This approach has been claimed as a therapeutic strategy to treat asthma and acute myeloid leukemia [114]. The second approach is the use of antisense oligonucleotides to down-regulate SCF expression [112, 115].

3.4.2 c-Kit

C-Kit is the receptor for SCF and is a member of the tyrosine kinase growth factor receptor family which is expressed on a variety of hematopoietic cells, including mast cells [116]. SCF-dependent activation of c-Kit leads to activation of a number of downstream signaling pathways (figure 2). These downstream pathways are critical for mast cell function and mast cell number. Blocking c-Kit or downstream pathways have been investigated as strategies for blocking c-Kit-mediated responses.

C-Kit has a large extracellular domain with five immunoglobulin-like domains, a single transmembrane span, and a long cytoplasmic tail containing a kinase domain which is split into two domains by an insertion region [106]. In addition, the intracellular domain has multiple tyrosine residues which are targets for phosphorylation. SCF binds with high affinity to c-Kit which induces dimerization of c-Kit and the initiation of its tyrosine kinase activity. This leads to phosphorylation of 9 tyrosines in the cytoplasmic tail [108]. Associating Src kinases such as Lyn and Fyn may also contribute to the phosphorylation [117]. The importance of c-Kit in mast cell function is exemplified by mutations that alter the

functionality of c-Kit. For example, when c-Kit is inappropriately activated such as by the substitution of valine for aspartic acid 816 (D816V) in the kinase domain, mastocytosis can occur [116].

Dimerization of c-Kit by SCF and subsequent autophosphorylation of tyrosines in the cytoplasmic domain of c-Kit initiates the activation of different critical signaling molecules. The phosphorylated tyrosines within the cytoplasmic tail of c-Kit serve as docking sites for Src homology 2 (SH2) domains of associating signaling proteins, including: 1) cytoplasmic adaptor molecules such as Grb2 and SHC; 2) Src kinases such as Lyn and Fyn; 3) signaling enzymes such as phospho-inositide 3-kinase (PI3K), and phospholipase Cγ (PLCγ). The activation of PI3K, in addition to the activation of the Ras/Raf/MAPK and the JAKs/STATs pathway, leads to the activation of a variety of transcriptional/survival factors (reviewed in [106, 118-122]). These latter signals are important, amongst others, for cytokine production, mast cell migration and survival (Figure 2). A recent study showed the importance of functional c-Kit for the development of neurofibroma in neurofibromatosis type 1-deficient mice. Moreover, pharmacologic inhibition of c-Kit activity with imatinib mesylate therapy resulted in a dramatic reduction in the tumor volume in a patient with plexiform neurofibroma [123].

3.4.3 Inhibition

Various tyrosine kinase inhibitors block c-Kit activity [120]. The most widely recognized compound blocking c-Kit catalytic activity is imatinib mesylate (also known as STI 571/Gleevec/Glivec). Imatinib mesylate blocks c-Kit signalling within mast cells and mastocytoma cells, preventing cell proliferation and survival [124]. Besides, Imatinib also inhibits Bcr-Abl and PDGF receptor tyrosine kinases, and is currently used for the treatment of patients with chronic myeloid leukemia (CML) [125], and gastrointestinal stromal tumors (GISTs) [126]. Nilotinib (AMN107) is an orally available signal transduction inhibitor of c-Kit, Bcr-Abl, and PDGF receptor (PDGFR) tyrosine kinases. Nilotinib inhibits both wild-type Kit and Kit carrying mutations, except of the D816V mutation and could therefore inhibit activation and proliferation of mast cells [127]. PI3K appears to be major signaling protein in tumors and is immediately downstream of c-Kit in a signaling pathway also known as the “survival” kinase pathway [128, 129]. Downstream of c-Kit are signaling pathways involving PI3K and other important kinases called AKT and mTOR. Currently, there are drugs in clinical trials that inhibit the PI3K downstream pathway. However, blocking PI3K may induce side effects such as inhibition of insulin function and heart-failure [130, 131].

A number of other compounds that are primarily considered to be multiselective tyrosine

kinase inhibitors could be used to inhibit mast cell proliferation and activation via c-Kit or other targets in the activation pathway:

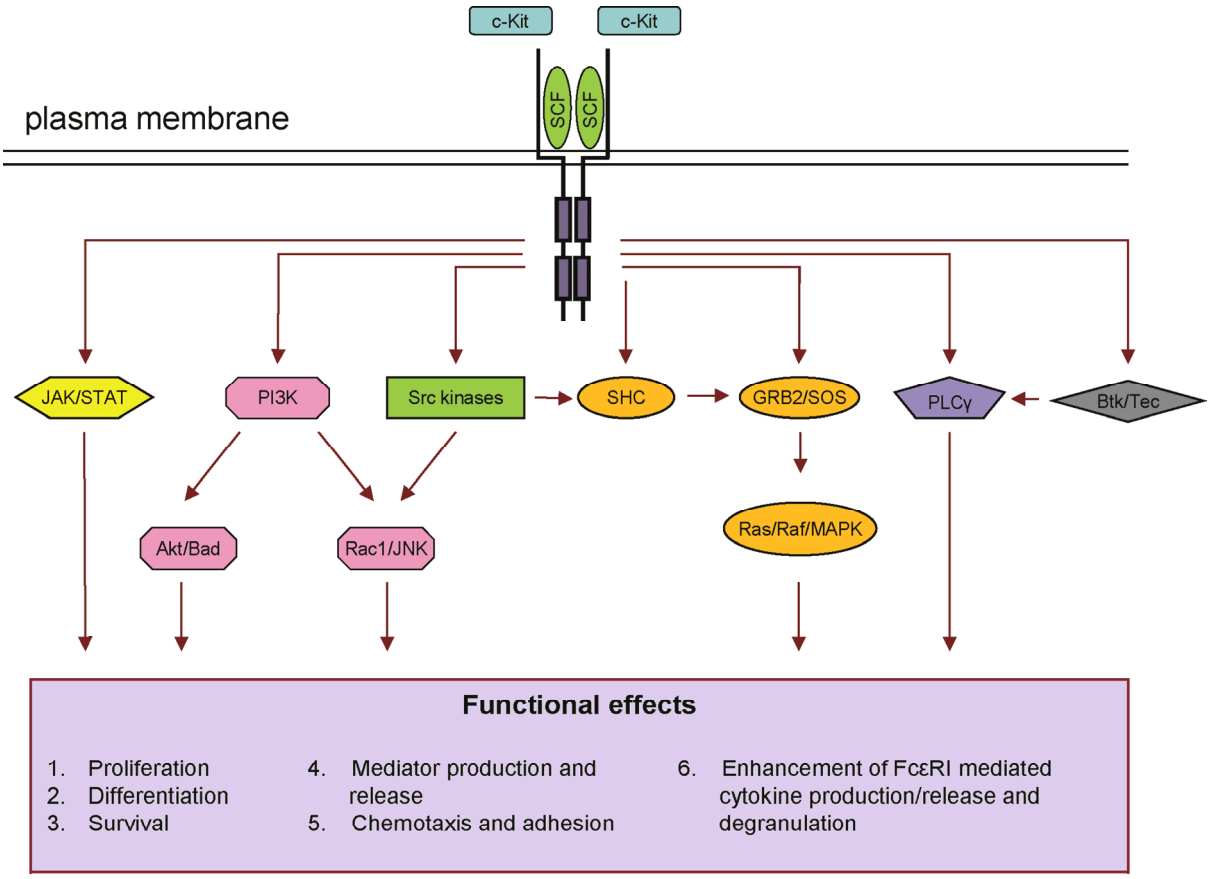

Figure 2. Schematic representation of major signaling cascades activated in mast cells after SCF binding to the c-Kit receptor. Binding of SCF rapidly induces receptor dimerization and subsequent autophosphorylation of tyrosine residues within its cytoplasmic tail. This leads to the activation of several signal transduction pathways, including the JAK/STAT pathway, PI3K, Src kinases, the Ras-Raf-MAP kinase cascade, and PLCγ. Activation of these pathways results in multiple functional effects, like increased cell proliferation, differentiation and survival. For detailed information about these signaling cascades, see [106, 118, 119, 121, 122].

1. Dasatinib is an orally bioavailable, synthetic small molecule-inhibitor of multiple tyrosine kinases, such as c-Kit, including the D816V mutated c-Kit, ABL, SRC, and PDGFR [132]. Dasatinib blocks proliferation of neoplastic mast cells via c-Kit [133]. IgE–mediated activation of basophils [134] is inhibited by dasatinib and a similar inhibitory effect on mast cells is expected.
2. Midosataurin is a synthetic multikinase inhibitor with potential anti-angiogenic and antineoplastic activities. Midostaurin inhibits c-Kit, VGFR2, PDGFR, and FMS-like tyrosine kinase 3 (FLT3), which may cause disruption of the cell cycle, inhibition of mast cell proliferation, and inhibition of angiogenesis [135, 136].

3. Hypothemycin is a resorrcyclic acid lactone antibiotic that was identified as active in a screen for inhibitors of T cell activation [137]. Hypothemycin is able to inhibit the phosphorylation of the classical MAP kinases ERK1/2. Hypothemycin also inhibits mast cell activation at the level of Btk and subsequent PLCγ1 activation and calcium mobilization [138, 139] .
4. MLN518 is a substance being studied in the treatment of some types of cancer. It is a type of tyrosine kinase inhibitor also called tadutinib. MLN518 inhibits cell proliferation by inhibiting different classes of c-Kit mutants by specific targeting the phophorylation of signal transducers and activators of transcription factor 3 (STAT3) [140].
5. PD180970 inhibits mast cell growth by blocking the Bcr-Abl kinase mediated STAT 5 signaling pathway. This compound is a potential therapeutic agent for chronic mylogenous leukemia [141].
6. Sunitinib is a multikinase inhibitor of VEGFR, PFGFR and FLT3. It has also been reported to inhibit c-Kit containing mutations in systemic mastocytosis and imatinib-resistant Kit mutations [142].
7. Enzastaurin is a type of serine threonine kinase inhibitor of protein kinase β (PKC β) which can be regulated by calcium-mobilization in mast cells. This kinase has been involved in regulation of mast cell activation and cytokine release [143, 144]. Enzastaurin may prevent cell growth and mast cell degranulation.
8. Semaxinib is an antitumor compound that inhibits c-Kit, VEGFR-2, and FLT3 [145]. Semaxinib inhibits the function of c-Kit by targeting Akt, ERK1/2 and STAT3 downstream signaling pathways and could therefore affect mast cell activation and growth [146].
9. Miscellaneous: Sorafenib is an oral tyrosine kinase inhibitor that inhibits the tyrosine kinase activity of Raf kinase (Raf-1, wild-type B-Raf, and B-Raf V600E), VEGFR, and PDGFR [147]. Pazopanib (GW786034) is another oral, multitargeted kinase inhibitor with specificity for c-Kit, VEGFR, and PDGFR. Both inhibitors are used as drugs in the treatment of renal cancer and are expected to also have inhibitory activity in mast cell activation [148].

4. Concluding remarks

As discussed in this review, mast cells can either be beneficial or detrimental to tumor development, which may depend on the stage of tumor development during which mast cells infiltrate, the communication with other cell types, or on the tumor microenvironment. The tumor promoting effects of mast cells are mainly mediated by secretion of pro-angiogenic

factors and by tissue remodeling, enhancement of tumor cell proliferation, and immunosuppression. However, many other inflammatory cells infiltrate into the tumor microenvironment. These cells, including tumor associated macrophages (TAMs), are also thought to have important roles in tumorigenesis [44, 47, 149, 150]. Since mast cells are capable of affecting chemotaxis, proliferation, and activity of many of these cell types, they might be critical regulators within the tumor microenvironment of inflammation and consequently of tumor growth. Besides, we discussed many therapeutic strategies that are available to inhibit mast cell activation and chemotaxis, although most therapeutics will also affect other immune cells. Further research in preclinical tumor models is necessary to delineate the value of the mast cell as a therapeutic target cell in the treatment of cancer.

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8

Immunoglobulin free light chains are linked with tumour-associated inflammation and poor cancer prognosis

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Abstract

Inflammation is an important pathophysiological component of various cancer types and its characterization has been shown to have prognostic potential. Current evidence indicates that tumour-infiltrating mast cells can promote tumour growth and angiogenesis. In this study, we show that immunoglobulin free light chains (FLCs) are involved in tumour progression through activating tumour-associated mast cells. Increased expression of FLC was observed in various human cancers including those of breast, colon, lung, pancreas, kidney and skin, and FLC expression co-localized with areas of mast cell infiltration. In a large cohort of breast cancer patients, FLC expression was shown associated with basal-like cancers with an aggressive phenotype. Moreover, lambda FLC was found expressed in areas of inflammatory infiltration and its expression was significantly associated with poor clinical outcome. Functional importance of FLCs was shown in a murine B16F10 melanoma model, where inhibition of FLC-mediated mast cell activation with a peptide antagonist strongly reduced tumour growth. Collectively, this study identifies FLCs as a possible important player in the pro-tumourigenic activation of mast cells. Blocking this pathway may open new avenues for the inhibition of tumour growth and might improve the prognosis of cancer.

Introduction

Inflammation is considered a critical component of tumour progression in various cancer types. The crosstalk between inflammatory cells and tumour cells provides a tumour microenvironment that favors proliferation, invasion, migration and metastasis (1, 2) . The mast cell is an important organizer of such inflammatory reactions (3). Through the release of cytokines and proteases, mast cells promote angiogenesis and tissue degradation and they function as important intermediates in regulatory T cell-induced tolerance (4-6). Many human tumours such as breast carcinoma, malignant melanoma, colorectal adenocarcinoma, and oral squamous cell carcinoma are infiltrated with mast cells at their periphery (5, 7-9). It has been shown that the number of mast cells correlates with an increase in intratumoural microvessel density, enhanced tumour growth and invasion, and poor clinical outcome (5, 7, 8, 10-12). Recent studies in various experimental tumour models have demonstrated an essential role for mast cells in tumour expansion (13-15). Nevertheless, this appears to be dependent on tumour localization (intra- versus peri-tumoural) and type of mast cell activation (piecemeal versus complete degranulation) (16, 17).

Mast cells can be activated by the crosslinking of cognate antigen to IgE antibodies that are bound to their high affinity receptor (FcεRI), which results in an inflammatory response. In addition, activation can be induced by many other signals, such as IgG, complement factors, bacteria, viruses, cytokines, inflammatory mediators, toll-like receptor ligands, and by T-cells (18). We have previously shown that immunoglobulin free light chains (FLCs) can activate mast cells in an antigen-specific manner (19), and along with IgE and IgG, FLCs are an important component of the humoral immune response to antigen exposure. In this study we investigated the presence and functional role of FLC in tumour-associated inflammation. We show that FLCs are expressed in human cancers of breast, colon, lung, pancreas, kidney and skin. Detailed analysis of a large cohort of breast cancer patients with long term clinical follow-up revealed that those patients with cancer of an aggressive phenotype showed an increased expression of lambda FLC in adjacent peri-tumoural stroma. The increased lambda FLC expression showed significant association with decreased survival, decreased disease free interval and a shorter time taken for metastasis formation. The functional importance of FLC was shown in a murine melanoma model, where it was found that inhibition of FLCs drastically inhibited tumour growth, supporting a possible crucial role of FLCs in the pro-tumourigenic activation of mast cells.

Results and Discussion

Localization of FLC expression in different human tumour types

The cellular localization of FLC expression was investigated in tissue microarrays (TMA) comprising human clinical cancer specimens of breast, pancreas, lung, colon, skin, and kidney tumours (20). Immunohistochemical staining was performed on TMA sections using the combination of anti-human kappa and anti-human lambda free light chain antibodies. Staining was evaluated in 642 samples of tumour tissue, adjacent tissue and normal control tissue (Supplementary Table 1) using a 3-point scoring method: +, isolated FLC-positive cells; ++, clusters of ≥ 10 FLC-positive cells; or +++, clusters of FLC-positive cells encompassing more than 10% of the tissue core (Figure 1A to C). FLC staining was noted in 201 out of 474 cancer tissue cores (42%) (Figure 1D and supplementary Table 1). Approximately 30% of the breast and pancreatic cancer samples showed FLC positive cells, whereas > 50% of lung, kidney, skin and colon cancer samples were FLC-positive. FLC staining was virtually absent in healthy tissue control samples, only 2/87 control samples were positive, which could possibly be related to other inflammatory conditions (21). Mast cells were detected using immunohistochemistry in essentially all tumour samples (143 out of 150 cases (95%)), supporting earlier observations of widespread mast cell infiltration in tumour tissues as reviewed by (22). FLCs (Figure 1E) and mast cells (Figure 1F) were present in the same regions.

Association of FLC expression and breast cancer

To further characterize whether FLC expression was associated with the manifestation of clinical disease of cancer, we analyzed kappa and lambda FLC expression separately in breast cancer tissue microarray slides from approximately 700 patients derived from the Nottingham Tenovus Primary Breast Carcinoma Series. This well characterized resource contains information on patients' clinical and pathological data (23). Protein expression of kappa FLC was predominantly localized in the cytoplasm of malignant breast cells and less frequently in inflammatory cells of the adjacent stroma. In contrast, strong lambda FLC expression was seen in stromal inflammatory cells but was only rarely seen in the cytoplasm of cancer cells (Figure 2A and B).

FLC positive patients showed no significant difference in age or tumour recurrence compared to FLC negative patients. Lambda but not kappa FLC expression was significantly increased in premenopausal patients (p=0.014). Kappa and lambda FLC protein expression showed significant positive correlations with increased tumour size, tumour grade and clinical stage

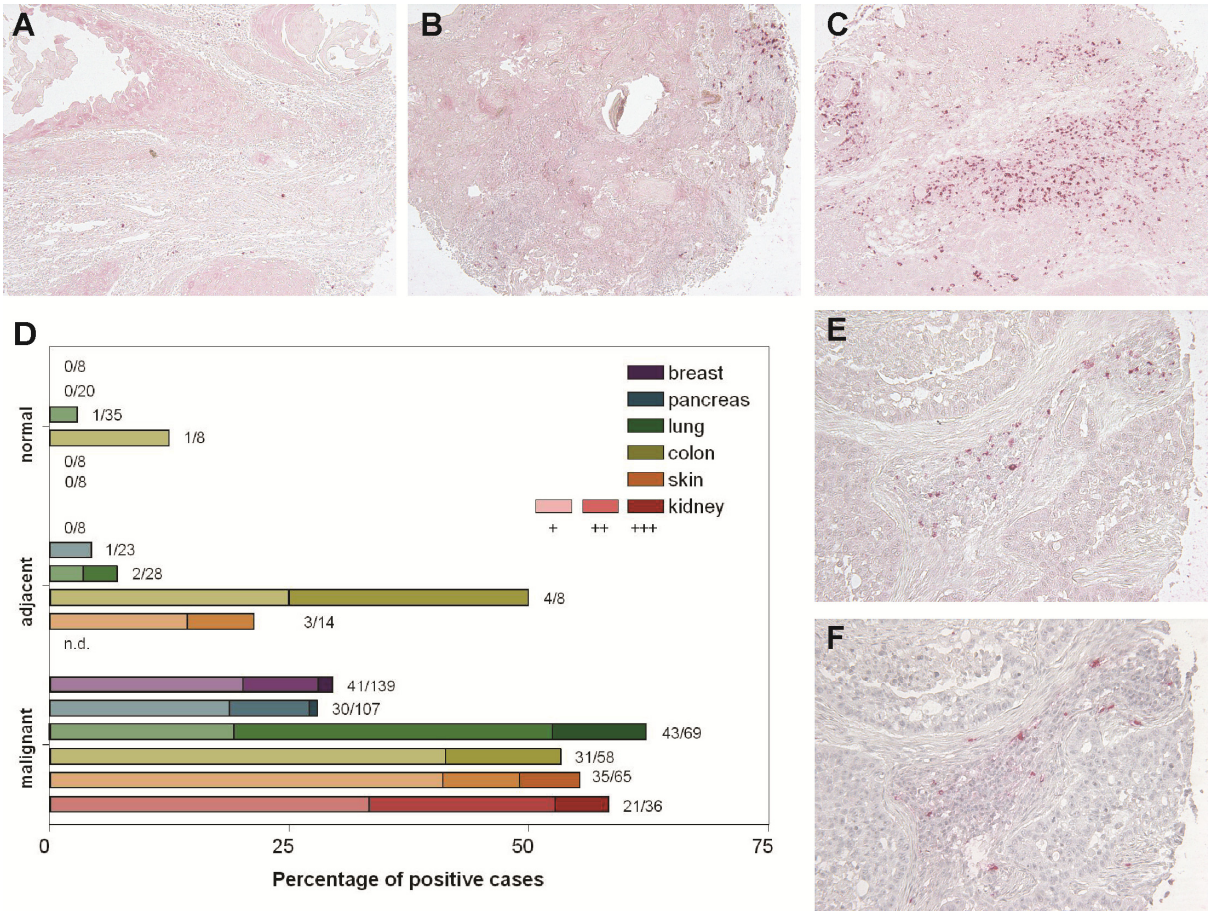

Figure 1. Immunoglobulin free light chains are expressed in breast, pancreas, lung, colon, skin and kidney cancer biopsies. (**A-C**) The FLC staining pattern was discriminated by three categories: +, isolated FLC-positive cells (**A**); ++, clusters of ≥ 10 FLC-positive cells (**B**); or +++, clusters of FLC-positive cells encompassing more than 10 % of the tissue core (**C**) (original magnification, x100)**. (D)** Percentage of FLC-positive malignant, cancer-adjacent or normal tissue biopsies (diameter 1.5 mm) from breast, pancreas, lung, colon, skin and kidney. The numbers next to each bar indicate the number of positive samples divided by the total number of individual patient biopsies examined. The color intensity of the bar corresponds to the degree of FLC staining. Biopsies were evaluated by two researchers who were blinded to the tissue status. (**E-F**) Expression of FLC (**E**) and infiltration of mast cells (tryptase staining) (**F**) were found in similar areas in tumour tissue (original magnification, x200).

(node involvement), poor NPI+ (Nottingham Prognostic Index), and vascular invasion (kappa $p<0.007$; lambda $p<0.002$). FLC positivity was under-represented in the low nuclear grade family of tumour types (24) including tubular cancers, but was increased in ductal and medullary type cancers ($p<0.001$). Medullary cancers belong to the family of triple negative (25) basal-like cancers which show features of necrosis and hypoxia and an aggressive phenotype (26). Kappa and lambda FLC expression showed significant negative correlations with tumour-relevant markers usually associated with favorable clinical outcome such as the hormonal markers estrogen and progesterone receptor and the apoptotic marker Bcl2 ($p<0.001$). Instead, significant positive associations were seen with markers of aggressive tumour phenotype such as basal-like markers including cytokeratin (CK) 5/6 and EGFR

(p<0.0001), and the proliferation marker MIB1 (p<0.002), and with mutated p53 and BRCA1 (p<0.0001), and HER2 (Kappa: p=0.004).

Multivariate Cox regression (hazards ratio: HR) was performed to test the independence of kappa and lambda FLC protein expression against established prognostic variables including tumour size, stage, grade, vascular invasion, chemotherapy and endocrine therapy, for predicting breast cancer specific survival (BCSS). FLC was not found to be an independent prognosticator of survival. But, Kaplan-Meier modeling of lambda FLC expression for clinical outcome in the full patient cohort (regardless of treatment) showed a significant correlation with decreased BCSS (χ^2=18.0, p<0.0001), disease-free interval (DFI) (χ^2=15.2, p<0.0001), tumour recurrence (χ^2=17.6, p<0.0001), and shortened time for metastases formation (DM) (χ^2=13.6, p=0.0002) (Figure 2C). No significant associations were found between kappa FLC protein expression and BCSS, DFI, tumour recurrence, or DM. Because kappa FLC is mainly found in the cytoplasm of malignant breast cells and not in the adjacent stroma , this indicates that the micro-localization of the expression of FLC in tumour tissue near peri-tumoural inflammatory cells might be of importance for aggravated tumour pathology and poor clinical outcome. The mechanism of the different localized expression of kappa and lambda FLC isoforms in tumour tissue needs further studies.

FLC play a crucial role in supporting tumour growth in a mouse melanoma model

In earlier work we have shown that mast cells can be activated by FLCs (19, 27). The functional importance of the association between FLCs and mast cells in tumour development and growth was further investigated in an *in vivo* mouse model. Using western blotting, we demonstrated the presence of FLCs in B16F10 melanoma generated subcutaneously in C57Bl/6J mice (Figure 3A). The tumour tissue contained monomeric (25 kDa) and dimeric (50 kDa) forms of FLC. B16F10 melanoma cells did not produce FLCs *in vitro* (data not shown), which suggests that local B-cells or plasma cells are a likely source of FLCs. B-cell infiltration and antibody production have been shown to precede premalignant transformation and to enhance tumour proliferation (28, 29).

Mast cell infiltration, a prominent feature of B16 melanoma models (30, 31), was also observed, especially at the tumour periphery (Figure 3B and C). The functional role of FLCs and mast cells in tumour growth in the B16F10 melanoma model was investigated both in wild type C57Bl/6J mice that possess normal numbers of mast cells and mast cell-deficient WBB6F1/J-Kit^{W}/Kit^{W-v} mice. After subcutaneous tumour cell inoculation, the average time for the tumours to become palpable was 7.7 ± 0.5 days in C57Bl/6J mice (mean ± SD, *n* = 20) and 7.5 ± 1.4 days for WBB6F1/J-Kit^{W}/Kit^{W-v} mice (mean ± SD, *n* = 20).

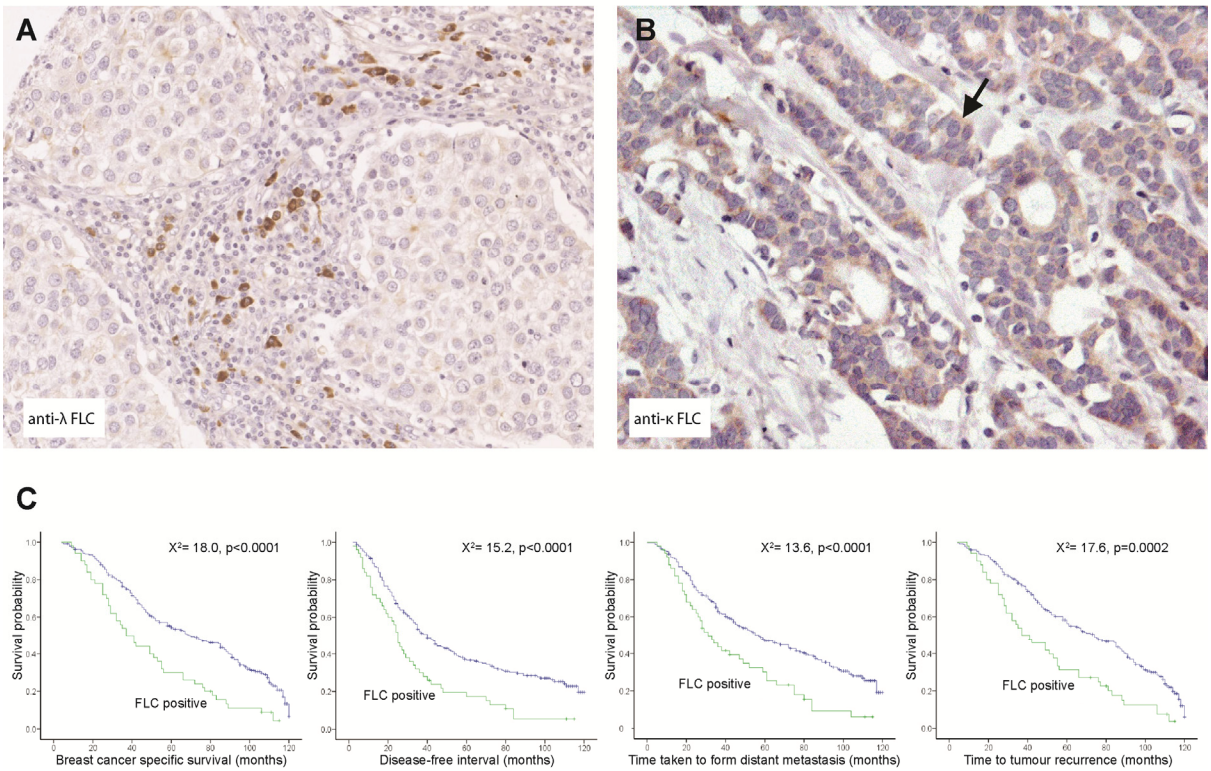

Figure 2. Lambda FLCs are found co-localized to regions of inflammation in breast tumours and their presence predicts breast cancer prognosis. (A) Lambda FLC protein expression was primarily localized to inflammatory cells located close to medullary breast cancer cells and in the cytoplasm of some cancer cells. (**B**) Kappa FLC protein expression (arrow) was mainly detected in the cytoplasm of ductal breast cancer cells (original magnification **A** and **B,** x100). (**C**) Kaplan-Meier plots showing patients with lambda FLC positive cells had reduced breast cancer specific survival, reduced disease-free interval, reduced time in forming metastases, and reduced time to tumour recurrence.

However, subsequent tumour growth was greatly reduced in WBB6F1/J-Kit^{W}/Kit$^{W\text{-}v}$ mice (P=0.03 comparing areas under the tumour growth curve), confirming a critical role for mast cells in B16F10 melanoma tumour growth (Figure 3D) (31). The role of FLC-induced mast cell activation in supporting the tumour growth was further investigated by using a specific peptide FLC antagonist (F991) (19, 27, 32). When tumours were palpable, the animals were treated intra-tumourally with weekly doses of 20 µg F991 or with vehicle (PBS) alone. In the wild type C57Bl/6J mice, F991 greatly reduced tumour growth when treated with F991. The first vehicle-treated wild type animals were culled at day 9 after the first treatment because they had reached the humane endpoint (tumour volume ≥ 1500 mm^3). At this time point, F991-treated wild type animals showed an average 61% reduction in tumour volume (p = 0.03) and a 49% reduction in growth (p = 0.02; total area under the tumour growth curve) compared to non-F991 treated control animals (Figure 3E). Four replicate experiments were performed and the volumes of F991-treated tumours were between 52 and 76% smaller than those of controls. The median time-to-reach-the-humane-endpoint in F991-treated wild type mice was also increased to 13 days compared to 10 days in vehicle-treated wild type

controls (p = 0.03) (Figure 3F). As expected, F991 did not affect tumour growth in mast cell-deficient WBB6F1/J-Kit^{W}/Kit^{W-v} (Figure 3E), nor did it affect the median survival time in mast cell-deficient animals (Figure 3F). Our previous studies demonstrated that FLCs mediate antigen-specific mast cell activation (19, 27) and the present findings implicate that FLCs may be crucial in the pro-tumourigenic activation of mast cells.

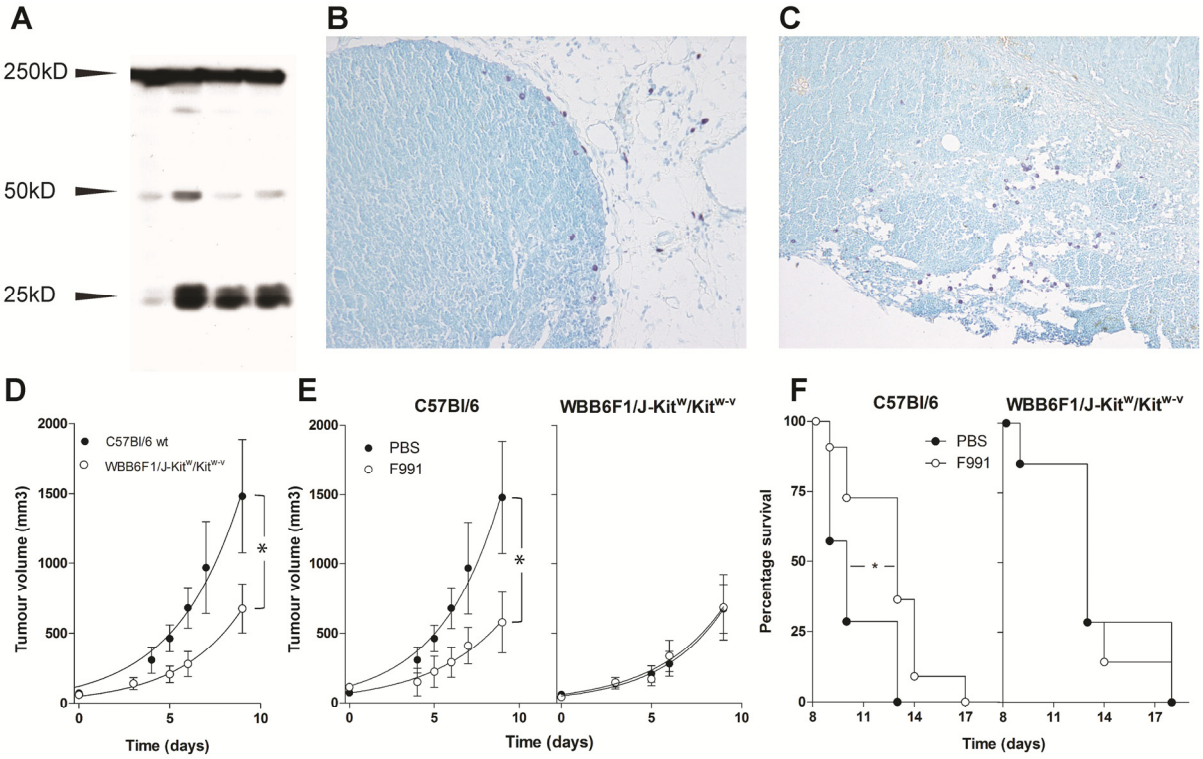

Figure 3. FLCs are responsible for mast cell activation supporting tumour growth of B16F10 melanoma. (**A**) Immunoblot analysis of FLC monomers (25 kD) and dimers (50 kD) in homogenized B16F10 tumour tissue. Each lane represents a tumour isolated from an individual animal. (**B-C**) Toluidine blue staining of mast cells in B16F10 melanoma tissue showing peritumoural and intratumoural mast cells. All of the melanomas were isolated from B16F10 inoculated wild type mice. **(D-F)** Mast cell deficiency and the FLC antagonist F991 attenuate B16F10 melanoma growth. **(D)** Tumour growth in PBS-treated wild type C57Bl/6 (closed circles) and mast cell-deficient WBB6F1/J-Kit^{W}/Kit^{W-v} (open circles) mice. (**E**) Tumour growth in wild type C57Bl/6 (left) and mast cell-deficient WBB6F1/J-Kit^{W}/Kit^{W-v} (right) mice treated weekly with 20 µg F991 (open circles) or PBS (closed circles) intratumourally. Data are the mean ± SEM, n = 10 animals/experimental group, * indicates p < 0.05 by comparing total areas under the curve (D and E). (**F**) Time-to-reach-humane-endpoint in wild type C57Bl/6 (left) and mast cell-deficient WBB6F1/J-Kit^{W}/Kit^{W-v} (right) mice treated weekly with 20 µg F991 (open circles) or PBS (closed circles) intratumourally. n = 10 animals/experimental group, * indicates p < 0.05. Effects of F991 were tested in 4 independent replicate experiments.

Taken together, our experiments provide a novel biological mechanism for the involvement of FLCs in tumour pathology. Inhibition of FLC-induced tumour-associated mast cell activation greatly reduced tumour growth in an experimental tumour model. In various human cancers including those of breast, colon, lung, pancreas, kidney and skin FLC

expression is found in areas of tumour-associated inflammation. Analysis of a large cohort of breast cancer patients showed association of FLC expression with an aggressive tumour phenotype such as hormonally-negative triple basal-like tumours. Our study suggests that analysis of FLC expression might have a prognostic capability in identifying cancer patients with poor clinical outcome and that interference with FLC-mediated mast cell activation may prove beneficial as a possible approach in cancer therapy.

Materials and Methods

Detection of FLC positive cells and mast cells in tissue arrays of different human tumour types

Tissue array slides from US Biomax (Rockville, MD, USA) were stained using the Envision G2 System/AP (rabbit/mouse; DakoCytomation, Cambridge, UK) according to manufacturer's instructions. Separate sections were stained for kappa and lambda FLCs simultaneously (Fκ-C8 and Fλ-G9 purchased from Dr. A. Solomon, TN, USA; both 1 µg/mL) and mast cell tryptase (clone AA1, DakoCytomation; 0.4 µg/mL).

Primary antibodies were diluted in Tris-buffered saline containing 0.1% Triton X-100 and 1% bovine serum albumin. For tryptase staining, tissue deparaffinization was followed by heat-induced epitope retrieval using citrate buffer (10 mM citric acid containing 0.05 % Tween-20, pH 6). Slides were counterstained with hematoxylin. Sections were viewed using an Eclipse TE2000-U inverted microscope with 4x and 40x objectives (Nikon). Images were analyzed using NIS elements BR 2.3 software (Nikon). FLC staining was differentiated as follows: – (absence of staining), + (isolated cells with positive staining for FLC), ++ (clusters of ≥ 10 cells with positive staining for FLC), or +++ (clusters of cells encompassing > 10 % of the tissue core with positive staining for FLC). Staining was assessed by two researchers who were blinded to the biopsy status.

Patient selection for detailed analysis of FLC expression in breast cancer

Tissue microarray (TMA) slides from the Nottingham Breast Cancer group were used in this study comprising approximately 700 patients from women aged 70 or less derived from the Nottingham Tenovus Primary Breast Carcinoma Series (1986 and 1999). This well characterized resource contains information on patients' clinical and pathological data including histologic tumour type, primary tumour size, lymph node status, histologic grade, and data on other breast cancer relevant biomarkers (33). Patients within the good prognostic group (Nottingham Prognostic Index (NPI) ≤3.4) did not receive adjuvant therapy (AT) (34). Hormonal therapy (HT) was prescribed to patients with ER-α+ tumours and NPI

scores of >3.4 (moderate and poor prognostic groups). Pre-menopausal patients within the moderate and poor prognostic groups were candidates for CMF (Cyclophosphamide, Methotrexate, and 5-Flourouracil) chemotherapy. Conversely, postmenopausal patients with moderate or poor NPI and ER-α+ were offered HT, while ER-α- patients received CMF if fit. Survival data including survival time, disease-free interval (DFI) and development of loco-regional and distant metastases (DM) were maintained on a prospective basis. Median follow up was 124 months (range 1 to 233). Breast cancer specific survival (BCSS) was defined as the time (in months) from the date of the primary surgical treatment to the time of death from breast cancer. DFI was defined as the interval (in months) from the date of the primary surgical treatment to the first loco-regional or distant metastasis.

This study was approved by the Nottingham Research Ethics Committee 2 under the title "Development of a molecular genetics classification of breast cancer". Patients consented to tissue samples being used for research purposes.

Immunostaining of human breast cancer tissue arrays

The localization and number of kappa and lambda FLC protein expressing cells was assessed in formalin fixed paraffin embedded (FFPE) tissue microarrays of breast cancer to characterize their association with cancer-relevant biological markers and with clinicopathologic features. The cut-offs used for categorizing the various biomarkers have been previously described (23). HER2 scoring was performed using the Hercept tests guidelines (DakoCytomation).

Immunohistochemistry (IHC) was performed using a DakoCytomation Techmate 500 plus (DakoCytomation) instrument with a linked streptavidin biotin technique and DAB chromogen as previously described (26). Primary antibodies were optimized on full face FFPE sections and TMAs of breast cancer tissue. Negative controls comprised omission of the primary antibody and substitution with an inappropriate primary antibody of the same Ig class. After deparaffinization, sections were subjected to microwave antigen retrieval using 0.01M citrate buffer, pH6 and then immunostained using the optimized 1:50 dilution of mouse monoclonal anti- kappa (Fκ-C8) and separately, anti-lambda FLC (Fλ-G9) Ab.

Staining intensity was subjectively assessed (DGP & HOH) for each marker according to a three point scoring system comprising: 0 (negative); 1 (weak); or 2 (strong) staining intensity confined to the malignant tissue or stromal inflammatory cells. Cases were scored without knowledge of patient outcome.

Univariate and Multivariate Statistics

The results have been reported according to REMARK criteria that establish a framework for reporting tumour marker prognostic studies (35). The association between FLC and other tumour-relevant markers was assessed using Chi square test. Association with clinical outcome including BCSS, DFI, DM formation, and local tumour recurrence was modeled using Kaplan-Meier plots (Version 15, SPSS Inc, IL, USA) with the log rank (Mantel-Cox) test. A p-value of less than 0.05 was deemed significant with 95% confidence intervals.

Animal studies

Detection of FLCs and mast cells in mouse B16F10 melanoma tissue

B16F10 melanoma tumours were grown subcutaneously in mice (36). In brief, 10^6 B16F10 cells cultured *in vitro* were injected subcutaneously into the flank of C57Bl/6J wild type or mast cell-deficient WBB66FI/J-Kit^{w}/$Kit^{W\text{-}v}$ mice. For mast cell detection, tumours were fixed in 10% buffered formaldehyde and embedded in paraffin. Deparaffinized sections were stained with toluidine blue solution. FLCs were detected by western blotting. Mouse tumours were homogenized and lysed with MT Cell lysis reagent containing a protease inhibitor cocktail. The lysed sample was centrifuged for 10 min at 20000 × *g* to pellet the tissue debris, and the protein supernatant was subjected to western blotting (iBlot; Invitrogen, Frederick, MD, USA). Horseradish peroxidase-labeled goat anti-mouse kappa light chain (0.1 µg/mL, SouthernBiotech, AL, USA) was used to immunostain the membranes.

Treatment with the peptide antagonist F991

C57Bl/6J wild type or mast cell-deficient WBB66FI/J-Kit^{w}/$Kit^{W\text{-}v}$ mice that received B16F10 cells via subcutaneous flank injection were monitored for tumour growth. At the time the tumour became palpable, 25 µl PBS containing 20 µg F991 or vehicle alone was injected in the tumour vicinity. Treatment was repeated weekly. Tumour growth was monitored by measuring the largest and smallest superficial diameters of the tumours using digital calipers. The tumour volume was calculated as follows: (0.52 x largest diameter) x (smallest diameter x smallest diameter). Animals were considered to have reached the endpoint of the experiment when the tumour volume measured ≥ 1500 mm^3.

Average tumour volumes and the total area under the tumor growth curves between F991 and PBS treated mice were compared using a Student's t-test. The differences in median time-to-reach-the-humane-endpoint of F991- and PBS-treated mice were analyzed using a Logrank (Mantel-Cox) test.

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Supplementary Results

Supplementary Table 1. FLC staining in biopsies of human malignant tissue in various organs compared to staining in tissue adjacent to the tumour and normal tissue.

Breast		**number**	**+++**	**++**	**+**	**-**
Malignant		**139**	**28**	**11**	**2**	**98**
	Metastatic carcinoma	26	6	4		16
	Invasive ductal carcinoma	65	17	5	2	41
	Lobular carcinoma	20	1	1		18
	Intraductal carcinoma	20	4	1		15
	Squamous cell carcinoma	4				4
	Lobular carcinoma in situ	4				4
Adjacent		8				8
Normal		8				8
Pancreas						
Malignant		**107**	**20**	**9**	**1**	**77**
	Ductal adenocarcinoma	89	19	9	1	60
	Adenosquamous carcinoma	3	1			2
	Islet cell tumour/carcinoma	11				11
	Metastatic carcinoma	4				4
Adjacent		23	1			22
Normal		20				20
Lung						
Malignant		**69**	**13**	**23**	**7**	**26**
	Adenocarcinoma	19	6	6	3	4
	Alveolar carcinoma	1		1		
	Squamous cell carcinoma	42	7	16	4	15
	Small cell undifferentiated carcinoma	7				7
Adjacent		28	1	1		26
Normal		35	1			34
Colon						
Malignant		**58**	**24**	**7**		**27**
	Adenocarcinoma	18	4	4		10
	Mucinous adenocarcinoma	14	6			8
	Carcinoid	2	1			1
	Metastatic carcinoma	20	10	3		7
	Papillary adenoma	4	3			1
Adjacent		8	2	2		4
Normal		8		1		7

Skin		**number**	**+++**	**++**	**+**	**-**
Malignant		**65**	**26**	**5**	**4**	**30**
	Squamous cell carcinoma	47	17	2	1	27
	Basal cell carcinoma	11	6	2	1	2
	Melanoma	7	3	1	2	1
Adjacent		14	2	1		11
Normal		8				8
Kidney						
Malignant		**36**	**12**	**7**	**2**	**15**
	Clear cell carcinoma	11	4	2	1	4
	Granular cell carcinoma	12	4	4		4
	Transitional cell carcinoma	20	4	1	1	7
Adjacent		n.d.				n.d.
Normal		8				8

FLC staining was analyzed in human clinical cancer specimens of different organs and different etiologies. Positive FLC staining pattern was discriminated by three categories: +, isolated FLC-positive cells; ++, clusters of ≥ 10 FLC-positive cells; or +++, clusters of FLC-positive cells encompassing more than 10 % of the tissue core. -, no FLC staining. n.d. not determined

9

Co-crosslinking of immunoglobulin free light chains can enhance IgE-mediated human basophil activation

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Abstract

Background: Basophils are increasingly being recognized as important players in the immune system via their release of diverse inflammatory mediators as well as the Th2 cytokines IL-4 and IL-13. Basophils can be activated by different mechanisms of which stimulation of receptors for IgE, complement, and fMLP are well known examples. In mast cells, another potential antigen-specific activating mechanism is demonstrated by crosslinking of immunoglobulin free light chains (FLCs). Presence of FLCs and a possible functional role on basophils is unknown.

Objectives: The presence and function of FLCs on human basophils was examined. In addition, the effects on basophil activation upon co-stimulation with FLC-crosslinking in combination with crosslinking of IgE, or addition of C5a or fMLP were analyzed.

Methods: FLC expression on basophils was analyzed by means of flow cytometry of whole blood from 4 adult donors. Additionally, whole blood was incubated with anti-IgE, C5a, or fMLP at different concentrations in the presence of different concentrations anti-FLC antibody. After incubation, basophil degranulation responses were determined by CD63 binding and analyzed by flow cytometry.

Results: FLCs are equally present on basophils from all tested donors. Crosslinking of FLCs alone did not result in basophil degranulation. In contrast, co-stimulation with IgE-crosslinking resulted in an enhanced basophil degranulation in 2 donors. This synergistic effect was absent when co-stimulating with C5a and fMLP.

Conclusions: FLCs are present on human basophils and FLC-crosslinking may enhance allergic activation of basophils. Since FLC concentrations are shown to be highly increased in many inflammatory disorders, this basophil activating capacity might have significant disease modifying potential.

Introduction

Basophils are rare blood-circulating leukocytes (normally <1% of circulating leukocytes) and are increasingly being recognized as critical players in the immune system (reviewed in [1-3]). Basophils contain only small amounts of proteases and cytotoxic proteins, but upon activation, they are able to release many inflammatory mediators such as histamine, LTC4, and T helper type 2 cytokines IL-4 and IL-13. Different mechanisms of basophil activation are known, of which stimulation of receptors for IgE, complement, and fMLP are well known examples [1]. Despite the fact that basophils are blood-circulating cells and are hardly present in normal tissue, they can rapidly infiltrate inflamed tissues during late phase responses [4,5]. Based on these characteristics and recent advances in the understanding of basophil functions, basophils are supposed to be important immunomodulatory as well as effector cells that contribute to type I hypersensitivity reactions, particularly during the late phase responses. Moreover, other functions have been ascribed to basophils, including protective immunity against ecto- and endoparasites and the induction of allergic immune responses via their capacity to act as antigen-presenting cells and initiate Th2 responses [1,3].

Basophils show several similarities to mast cells, such as the expression of the high-affinity IgE receptor (FcεRI). Crosslinking of this receptor upon binding of antigen to membrane-bound IgE results in the release of inflammatory mediators that are in part common for both cell types [6]. Another potential antigen-specific mast cell-activating mechanism is mediated by crosslinking of immunoglobulin free light chains (FLCs) [7]. FLCs (either kappa or lambda isotype) are B cell products that are produced in excess over heavy chains and secreted without being incorporated into complete antibodies. As a consequence, FLCs can be found in serum, but also many other body fluids like urine, nasal secretions, bronchial alveolar lavage fluid, and synovial and cerebrospinal fluid [8-12]. Increased concentrations are found in several disorders, such as rhinitis, asthma, atopic dermatitis, rheumatoid arthritis (RA), and multiple sclerosis (MS). Interestingly, correlations are found between FLC concentrations and disease activity scores in RA and MS [10,11,13-16]. Current knowledge on the role of basophils in these disorders is limited, although some studies indicated the association of basophils with skin diseases, and allergic asthma and rhinitis, in which it was shown that basophil numbers increased during the late phase response upon allergen challenge [17-20].

Until now, little is known about the effects of FLCs on other cells than mast cells. In this study, we examined the presence and function of FLCs on human basophils. In addition, we examined the effects on basophil activation of co-stimulation with FLC-crosslinking in combination with crosslinking of IgE, or basophil activation by C5a or fMLP.

Materials and Methods

Blood samples

Whole blood was collected in sodium heparin tubes (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) from 4 healthy donors upon informed consent and was immediately processed. Whole blood was washed twice with excess phosphate buffered saline (PBS) and resuspended in RPMI 1640 medium (Lonza, Verviers, Belgium) to the same volume as the original blood volume.

Table 1. Baseline characteristics of blood donors.

	Donor 1	Donor 2	Donor 3	Donor 4
Age (year)	52	26	48	27
Sex	female	male	male	male
Allergy	grass pollen	grass pollen	house dust mite	grass pollen

Basophil activation

75 µL of whole blood aliquots (washed) were incubated with equal volumes of RPMI 1640 + IL-3 alone (R&D Systems Abingdon, UK; final concentration 1 ng/mL), or with the addition of goat anti-human IgE (KPL, Gaithersburg, MD, USA), complement factor C5a (Sigma-Aldrich, Zwijndrecht, The Netherlands), or N-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma-Aldrich). All conditions (anti-IgE, C5a, and fMLP) were tested at large concentration ranges and were performed with and without the addition of different concentrations of mouse anti-human kappa and lambda FLC (an antibody mix was used containing equal quantities of anti-kappa FLC (Fκ-C8) and anti-lambda FLC (Fλ-G9) (obtained from Dr. A. Solomon, TN, USA), and anti-kappa FLC (clone 4C11) and anti-lambda FLC (clone 3D12; Abcam, Cambridge, UK). Isotype control antibodies (IgG1, clone MOPC-21, Biolegend, CA, USA; IgG2a, clone MAB003, R&D Systems) were included as a control for the anti-FLC antibodies. After 30 minutes incubation at 37°C the reaction was stopped using 25 µL of cold PBS supplemented with 20mM EDTA.

Flow cytometry

To analyze basophil activation, cells were stained for the membrane expression of CD203c, CD123, and CD63 by addition of 25 µL of antibody mix containing the following antibodies: mouse anti-human CD203c-APC, 1:100, clone NP4D6; mouse anti-human CD123-FITC, 1:200, clone MEM-259; mouse anti-human CD63-PE, 1:100, clone 6H6, all from Biolegend,

CA, USA, diluted in PBS + 2mM EDTA + 0.5% BSA (staining buffer). Samples were incubated for 30 minutes in the dark on ice and subsequently washed in staining buffer. After red cell lysis using 2.5 mL FACS Lysing Solution (BD Biosciences, Breda, The Netherlands) per sample for 15 minutes, cells were resuspended in 200uL staining buffer containing 0.5% paraformaldehyde, and basophil activation was assessed by flow cytometry using a FACSCalibur (BD Biosciences). Basophils were identified as $CD123^{+}CD203c^{+}$ and their activation was quantified by the percentage of $CD63^{+}$ basophils.

To assess the presence of FLCs on basophils, washed whole blood was incubated with 20 µg/mL anti-human FLC antibody (mix as described above) together with goat anti-human IgE-FITC (1:50, KPL). Staining procedures were similar as described above, but an additional second incubation step with goat anti-mouse-IgG-APC (1:50, clone Poly4053, Biolegend) was included for FLC detection. Additional samples were stained for CD203c and CD123 to verify high IgE expression as a basophil marker. Blot overlays were produced using CellQuest software (BD Biosciences).

Results

Immunoglobulin free light chains are present on human basophils

Cells from (washed) whole blood were stained with a combination of anti-kappa and anti-lambda FLC antibodies to analyze the presence of FLCs on basophils. Basophils were identified by high IgE (FITC) expression on cells located in the lymphocytic cell population based on the forward and side scatter (Figure 1A and B; representative plots are shown). Using an additional double labeling for the basophil markers CD123 and CD203c we showed that the high IgE-expressing cells were indeed all basophils, since cell numbers were comparable for all donors (table 2). Subsequently, the presence of FLC on the high IgE-expressing basophils was found to be highly significant in all donors. Compared to isotype control staining (<5.0% of basophils was positive for the secondary antibody in all donors, representative plot is shown in figure 1C), there is a clear shift when using FLC specific antibodies in all donors (>90.0% of basophils was positive for the secondary antibody in all donors, representative plot is shown in figure 1D). Blot overlays illustrating these data for each donor are shown in Figure 1E-H for donor 1 to 4, respectively. Percentages indicate the cells that are positive for FLC staining as indicated in panel D.

IgE-mediated basophil activation is enhanced upon co-crosslinking of FLCs

Since clear FLC binding on basophils was found in all 4 donors, we analyzed its functional role in basophil activation. Therefore, we started with the addition of different concentrations of anti-FLC antibodies to achieve crosslinking, but no effect on CD63 expression was found

Table 2. Total basophil counts per donor and %CD63 positive cells after basophil co-stimulation.

		Donor 1		Donor 2		Donor 3		Donor 4	
Number IgE-high+ cells per 100.000 cells		1171		330		854		697	
Number CD123+CD203c+ cells per 100.000 cells		1149		352		845		696	
%CD63 positive cells after basophil co-stimulation									
		Isotype	**Anti-FLC**	**Isotype**	**Anti-FLC**	**Isotype**	**Anti-FLC**	**Isotype**	**Anti-FLC**
Anti-IgE (µg/mL)	**0**	0.2	0.6	0.2	0.8	0.0	0.0	0.0	0.3
	0.037	8.0	16.0	0.7	7.1	19.6	18.9	0.7	1.8
	0.11	22.1	33.2	11.1	21.8	28.3	24.7	2.9	5.4
	0.33	49.8	58.4	29.3	45.0	25.3	21.7	9.0	13.3
	1	34.8	38.2	23.6	25.1	15.8	8.9	4.9	4.8
C5a (nM)	**1**	0.7	1.0	0.2	0.9	1.3	2.3	0.4	0.3
	10	38.5	38.6	0.3	0.5	15	26.4	22.9	23.1
	100	40.1	42.1	1.0	0.3	39.3	31.0	25.9	24.3
fMLP (µM)	**0.001**	0.1	0.4	2.2	1.0	1.3	1.0	0.0	0.0
	0.01	6.6	7.4	28.4	34.8	11.4	12.0	29.4	28.7
	0.1	27.1	26.9	49.4	50.6	22.1	22.0	46.1	48.1
	1	28.3	36.5	55.6	52.5	23	24.1	46.0	44.5

In the lower part of the table the percentage of $CD63^+$ basophils is shown after stimulation with anti-IgE, C5a, or fMLP in combination with either isotype control or anti-FLC antibodies (both 5 µg/mL).

(data not shown). Subsequently, we combined different basophil stimulating agents in combination with anti-FLC antibodies to study the possibility that FLCs only influence basophil activation once they are co-activated. Crosslinking of IgE resulted in basophil activation in all donors, although the degree of activation was highly donor-dependent (Figure 2, left graphs). In 2 of the 4 donors we found a significant synergistic activating effect upon co-stimulation with anti-FLC in IgE-mediated basophil activation (Figure 2A and B right graphs). Compared to the combination of anti-IgE and isotype controls, an additional

absolute increase in CD63-positive basophil numbers of up to 11.1% and 15.7% was found after co-crosslinking of FLCs in donors 1 and 2, respectively.

Especially at low anti-IgE antibody concentrations, the synergistic effect of FLC-crosslinking is more pronounced when analyzing the relative increase in CD63 expression compared to co-stimulation with the isotype control. For example, 0.037ug/mL anti-IgE together with isotype control resulted in 8% CD63 positive cells in donor 1, whereas co-crosslinking of FLC using 5ug/mL antibody at similar anti-IgE concentrations resulted in 16% CD63 positive basophils. This is an absolute increase of only 8% but a 2-fold relative increase (200%).For donor 2 this increase was even 10-fold (isotype control: 0.7%; anti-FLC: 7.1% CD63 positive cells). The effect of FLC crosslinking in combination with anti-IgE on basophils from donors 3 and 4 was only minimal (Figure 2C and D). At some anti-FLC concentrations, an inhibitory effect on basophil activation was found in all donors, although this was much less than the activating effect seen in donor 1 and 2. In all donors we found an inhibitory effect on IgE-mediated basophil activation upon co-incubation with isotype control antibodies independent of antibody concentration and donor.

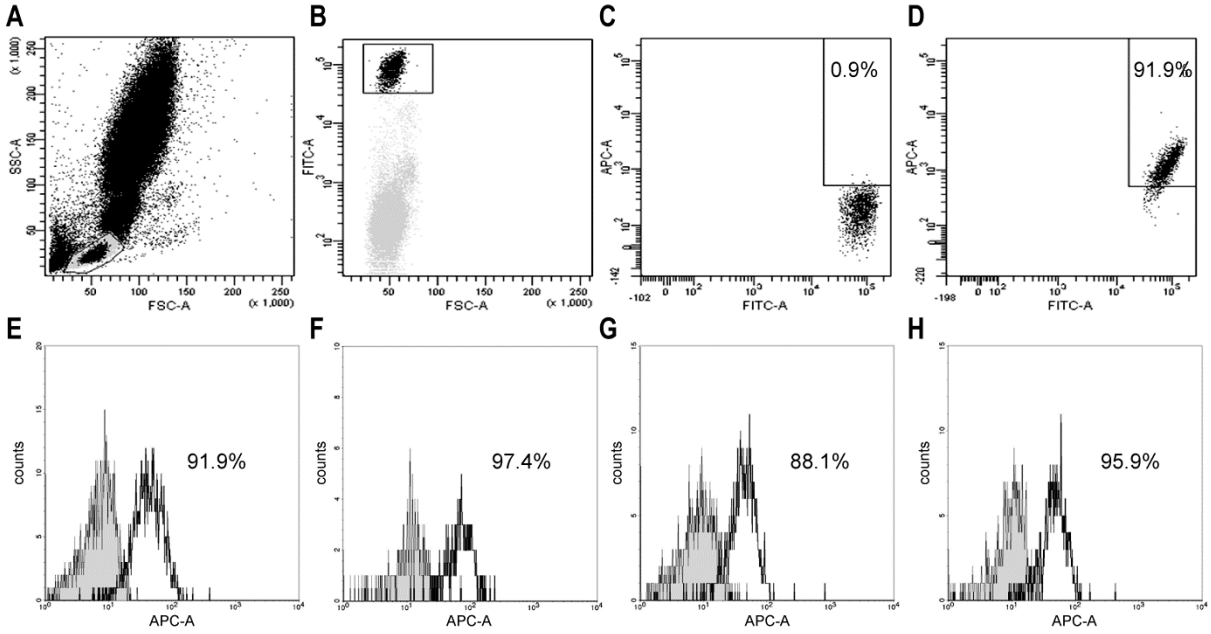

Figure 1. FLC expression on human basophils. Lymphocytic cells in washed whole blood were gated (**A**) and IgE expression (FITC) on this population was analyzed as a marker for basophils (**B**). Binding of mouse isotype control antibody (**C**) or mouse anti-human-kappa and lambda FLC antibody (**D**) was analyzed in these high IgE^+ cells by using a secondary goat anti-mouse IgG antibody (APC). Dot plots shown are from donor 1. **D,** Histogram overlays of panels C and D are shown for all donors (**E-F**, donor 1-4, respectively). filled histograms: isotype control, black histograms: anti-FLC. Percentages indicate the fraction of cells that show a high APC signal. FLC: immunoglobulin free light chain, FSC: forward scatter, SSC: side scatter, FITC: fluorescein isothiocyanate, APC: allophycocyanin.

Basophils from all donors responded to stimulation using C5a and fMLP, except for donor 2 which did not respond to C5a. In contrast to the synergistic effect of co-stimulation with anti-FLC on IgE-mediated basophil activation, this effect was not detectable in C5a- or fMLP-mediated basophil activation (table 2).

Discussion

In this study we demonstrate that FLCs bind to human basophils and that crosslinking of membrane-bound FLCs enhances IgE-mediated basophil activation as measured by CD63 membrane expression. Crosslinking of membrane-bound FLCs alone however, did not result in increased CD63 expression on basophils. Interestingly, all donors showed comparable FLC positivity, but clear inter-individual differences in the outcome of crosslinking of FLCs were observed. In 2 out of 4 donors, we found a synergy between IgE and FLC-induced basophil activation. This synergism seems restricted to stimulation by IgE, since we did not observe enhanced C5a- or fMLP-induced basophil activation by FLC crosslinking.

The finding that crosslinking of membrane-bound FLCs only influences basophil activation upon co-stimulation with anti-IgE corresponds with our recent findings on the effect of FLCs on mast cell activation [21]. Using different primary cultured bone marrow-derived mast cells and mast cell lines, including RBL-2H3 cells which also show features of basophils, it was shown that combined crosslinking of IgE and FLC resulted in a synergistic mast cell activation leading to degranulation and IL-6 production. In both studies, such synergistic effect was most noticeable at suboptimal IgE-mediated cell activation (up to 10-fold increase upon co-stimulation with anti-FLC). In contrast, at supraoptimal stimulating conditions (1 µg/mL anti-IgE), the synergistic effects of FLC-crosslinking are abolished in donor 1 and 2. An inhibiting effect on IgE-mediated activation might even be detected in donor 3 using these conditions. Future studies have to demonstrate whether similar effects are seen after crosslinking with antigen instead of anti-IgE. One possible mechanism explaining these findings is that FLC-mediated signaling is sensitive to the action of src homology 2 domain-containing inositol 5' phosphatase (SHIP). SHIP is highly activated after supraoptimal anti-IgE triggering leading to inhibited basophil activation [22]. Moreover, it was shown that non- and moderate responders to IgE crosslinking have a higher maximum SHIP phosphorylation. Consequently, differences in this SHIP phosphorylating capacity might underlie inter-individual differences in the effect of FLC-crosslinking since the two non-responders (figure 2C and D) show lowest optimal IgE-mediated basophil activation.

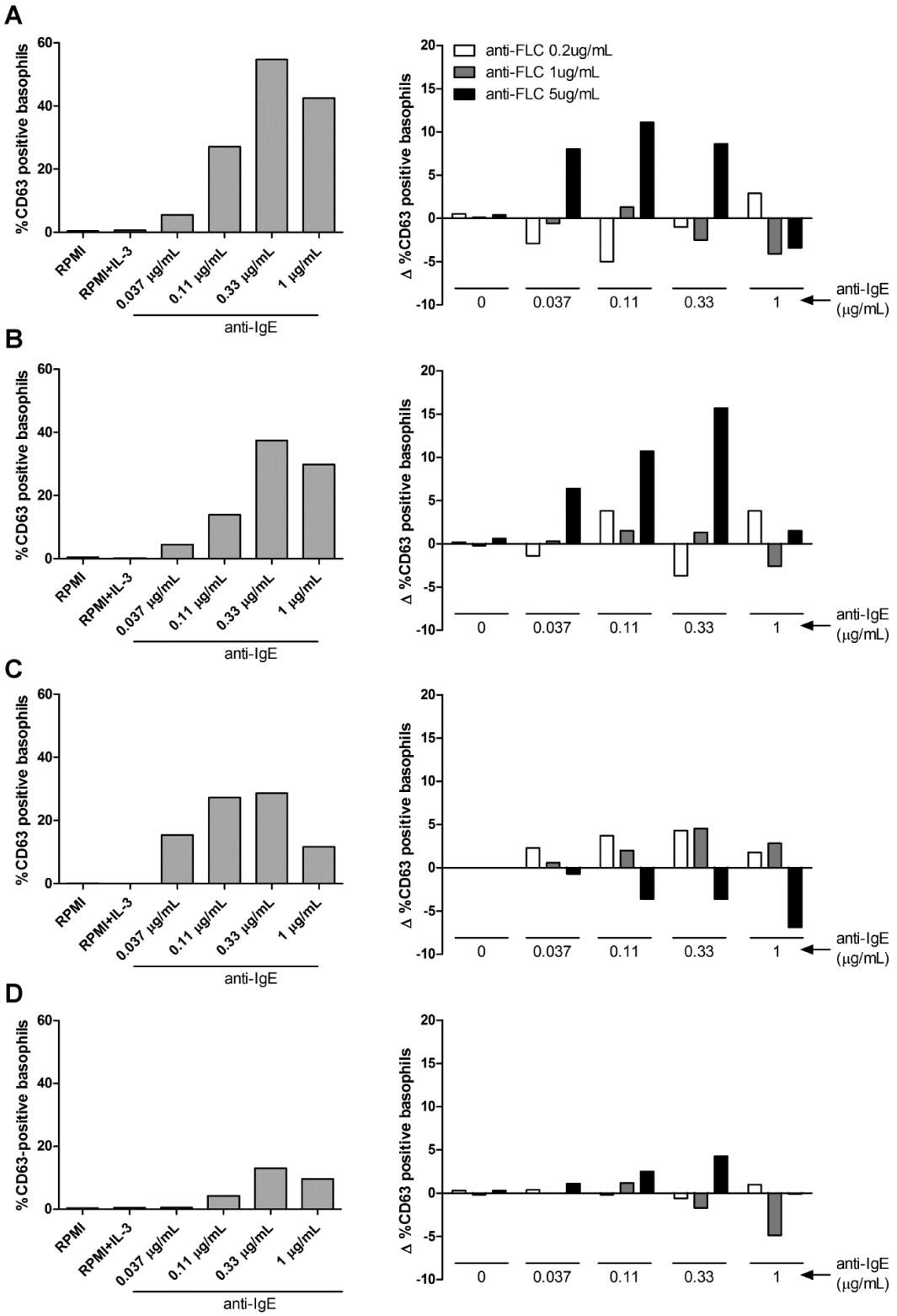

Figure 2. Crosslinking of FLCs modulates IgE-mediated basophil activation. Washed whole blood from 4 different donors was activated using different concentration of anti-IgE in combination with different concentrations of anti-kappa and anti-lambda FLCs (donor 1-4: **A-D**, respectively). Left panels: percentage of CD63 expressing basophils ($CD123^{+}CD203c^{+}$ cells) before and after IgE-mediated activation per donor. Right panels: The absolute difference (Δ) in percentage of CD63+ basophils between cells stimulated with anti-IgE in combination with anti-FLC and cells stimulated with anti-IgE in combination with isotype control. FLC: immunoglobulin free light chain.

The conditions in which low anti-IgE concentrations are used might be a realistic reflection of a physiologic situation when only part of the IgE present on basophils is crosslinked by one particular antigen. Relatively high anti-FLC antibody concentrations were required to observe the synergistic effect of co-stimulation by FLC-crosslinking on basophil activation. This may be due to a less effective crosslinking of membrane-bound FLC by the FLC-specific monoclonal antibodies as compared to the polyclonal anti-IgE antibody used in this study. Less comparable to the physiologic situation, in this study we analyzed washed blood. This was done to make basophil activation more sensitive and to exclude other interfering signals from serum proteins including unbound immunoglobulins. No effects were seen on basophil activation after FLC crosslinking alone, whereas anti-IgE did result in basophil activation in all donors, emphasizing the specificity of the used anti-FLC antibodies for light chains that are not incorporated into complete antibodies (e.g. in the membrane-bound IgE).

Synergistic effects on IgE-mediated basophil activation are also described for IgG. In a passive mouse model for systemic anaphylaxis it was shown that relatively small doses of antigen (ovalbumin) could induce severe anaphylaxis when mice had been passively sensitized with antigen-specific IgE and IgG [23]. In contrast to mouse basophils however, human basophils cannot be activated through IgG receptors because of the inhibition by IgG-mediated triggering via FcγRIIb receptors [24-26]. In this study, FcγRIIb-mediated inhibition of basophil activation might also be responsible for the (slight) inhibitory effect observed on IgE-mediated basophil activation by the addition of monomeric isotype control IgG [27]. Other factors shown to synergistically enhance basophil degranulation in response to IgE-crosslinking are IL-3 [28] and IL-33 [29,30]. Similarly, IgE-mediated mast cell degranulation and cytokine production can be influenced by co-stimulation of several receptors, such as growth factor receptors (including the SCF receptor, KIT), Toll-like receptors, and specific G protein-coupled receptors (GPCRs) [31]. The fact that a synergistic effect of FLC-crosslinking is only observed upon co-stimulation with anti-IgE, and not with C5a or fMLP (both GPCRs), suggests that IgE- and FLC-receptors may share common intracellular events such as recruitment and activation of tyrosine kinases, which can cooperate to increase basophil activation.

In general, two types of basophil activation can be distinguished, anaphylactic degranulation (AND) and piecemeal degranulation (PMD) [32]. However, in this study we analyzed CD63 expression, which has been shown to be more associated with the AND type [33]. Since basophils were primed with IL-3 before activation, subtle differences in CD203c expression (as a measure of PMD) could not be analyzed. Additional analyses of CD203c in similar experiments without IL-3 priming, and the analysis of cytokine and histamine release, which

can also be released by the process of PMD, would add further insight in the effects of FLC-crosslinking on basophil activation.

During recent years we showed that FLCs are involved in mast cell dependent experimental models for contact hypersensitivity, asthma, cow's milk allergy, and IBD [7,14,34-36]. Interestingly, in cow's milk allergy allergic sensitization by different antigens led to remarkable differences in IgE and FLC production [35]. Moreover, depletion of CD25-positive cells during sensitization with whey protein, switched the production of IgE into FLC in mice [36], while in cow's milk allergic children increases in total serum FLC, but not in IgE, are found [35]. FLCs recognize and bind antigen although with an overall lower affinity than most IgE antibodies (Groot Kormelink et al. Curr Pharm Des. 2011, *in pres*). Nevertheless, it has been shown that low-affinity antibodies can significantly enhance basophil degranulation upon co-stimulation with high or moderate affinity antibodies [37]. Therefore, it can be speculated that in allergic subjects this synergism between FLC and IgE could lead to degranulation responses even when antigen is at lower dose than threshold.

In conclusion, this study provides evidence that crosslinking FLCs may enhance allergic activation of basophils. Future studies using a specific FLC antagonist (F991) might give more insight in the contribution of FLC-crosslinking to basophil activation upon addition of specific activating antigens. Furthermore, involvement of basophils, and the influence of FLCs on its activation, has to be addressed in disorders in which (local) FLC concentrations are increased.

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10

Immunoglobulin free light chain monomers and dimers are differentially secreted by plasma cells and are functionally distinct

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Abstract

Background: Immunoglobulin light chains are produced by B lymphocytes in excess over heavy chains and are secreted in the circulation as immunoglobulin free light chains (FLCs). Regulatory mechanisms involved in the FLC production and secretion are largely unknown.

Objectives: To investigate whether FLC production is dependent on immunoglobulin class switching and if FLC production is increased in preclinical models associated to autoimmunity as also observed in human.

Methods: To analyze the relation between FLC and immunoglobulin subclass expression, serum FLC and immunoglobulin expression was analyzed in wild-type C57Bl/6 mice and Btk-, AID-, and CD40L- knockout (KO) mice. To analyze FLC expression in preclinical autoimmune models, serum from FcγRIIb KO mice and transgenic CD19-hBtk^{WT}, MHCII-hBtk^{WT}, Bcl-2, and MRL/Lpr mice was investigated. In addition, trinitrophenol (TNP)-specific FLCs were generated from TNP-specific mouse IgG and subsequently separated into FLC monomers and dimers. Antigen-binding of both isoforms was analyzed by using ELISA and western blotting.

Results: AID KO mice, which only produce IgM, almost exclusively produced FLC monomers whereas Btk KO mice, which selectively lack IgM and IgG3 expression, predominantly produced FLC dimers. CD40L KO mice did produce both FLC isoforms. It was shown that FLC dimers had significantly higher antigen-specific binding affinity than FLC monomers. Knockout and transgenic mouse strains associated with autoimmunity showed increased FLC monomer, FLC dimer, and immunoglobulin production.

Conclusions: FLC monomers and dimers can be differentially secreted suggesting the absence of an equilibrium balance between both isoforms. Moreover, this differential secretion appears to be class switch dependent. A preferential secretion of FLC dimers may be of functional importance because of their higher antigen-binding affinity compared to FLC monomers.

Introduction

B cells are responsible for maintaining humoral immunity which can be divided in innate-like responses and adaptive responses. The innate-like humoral response involves B1 cells and marginal zone (MZ) B cells that respond most rapidly to antigen by producing low affinity IgM and IgA antibodies. Both B1 and MZ B cells are mainly activated in a T cell independent (TI) way. In contrast, the late adaptive humoral response is T cell dependent (TD) involving conventional B2 (follicular; FO) cells that undergo a germinal center (GC) reaction in the spleen or lymph nodes [1-3]. During these GC reactions, multiple cycles of B cell proliferation, somatic hypermutation of the variable regions, and apoptosis result in the selection of only those B cells that produce high affinity antibodies. Moreover, immunoglobulin class switch recombination (CSR) takes place during these reactions [4]. Two proteins critically involved in germinal center formation, somatic hypermutation and class switching are CD40L (expressed on surface activated T cells) and activation induced cytidine deaminase (AID) in B cells [5, 6].

Intact immunoglobulins (Igs) consist of two identical heavy chains and two identical light chains which are covalently linked in heavy and light chain pairs. Both heavy and light chains have a C-terminal constant region and an N-terminal variable region, the latter containing the antigen binding complementarity-determining regions (CDRs) that are subject to somatic hypermutation. Next to the process of somatic hypermutation during the GC reaction, random rearrangement of a multitude of Ig variable (V), diversity (D), and joining (J) gene segments for heavy chains, and V and J segments for light chains are important for maintaining the diversity of variable regions [6].

Mammalian B cells can produce two types of light chains, either kappa (κ) or lambda (λ) [7], although the ratio in which both types are produced varies significantly among species (~50-75% κ production in human, ~95% κ in mice [8, 9]). The production of heavy and light chains is independent of one another and on average there is an excess production of light chains of up to 40% as compared to heavy chains [10-15]. In contrast to heavy chains, light chains can be secreted by B cells without being incorporated into immunoglobulins under physiological conditions, leading to the presence of unbound free light chains (FLCs) in the extracellular environment and in many body fluids, including the serum. In several different inflammatory disorders, including allergies and autoimmune diseases, an increased FLC production was observed systemically and at local sites of inflammation (reviewed in Groot Kormelink et al. Curr Pharm Des. 2011, *in press*). In all body fluids, κ and λ FLCs predominantly exist as monomers (FLCm; 22-27 kD) and covalently and non-covalently bound dimers (FLCd; 44-55 kD), but also polymeric forms of κ and λ FLC occur.

At this moment only little is known on the control of FLC production and secretion. In a study using neoplastic B cells that were isolated from patients suffering from different types of B lymphocytic diseases, it was shown that the amount of FLC produced is dependent on the degree of B cell stimulation [15]. Only light chain production was observed by unstimulated mature B cells, in contrast to a more balanced light chain/heavy chain production after induction of plasma cell differentiation. This was supported by the finding that small cell populations of the human spleen expressing IgM and IgD at the cell surface, resembling unstimulated mature B cells, produce the highest excess of light chain over heavy chain [12]. Furthermore, stimulation of cell cultures with either pokeweed mitogen (PWM) or lipopolysaccharide (LPS) resulted in a shift towards a more balanced light chain/heavy chain production [12, 16].

Since it has been shown that FLCs can exert several biological functions, including antigen-specific mast cell activation [17-19], it is of great interest to understand putative regulatory mechanisms involved in FLC production other than B cell activation state. In this study, we analyzed whether FLC production is class switch dependent by comparing FLC production in wild-type mice, and Btk-, AID-, and CD40L-deficient mice. Bruton's tyrosine kinase (Btk) is a cytoplasmic protein tyrosine kinase that is essential for B cell differentiation. Btk-deficiency leads to a moderate reduction in peripheral B cell numbers, absence of peritoneal $CD5^-$ B1 cells, overrepresentation of immature $IgM^{high}IgD^{low}$ and a specific deficiency of $IgM^{low}IgD^{high}$ mature B cells resulting in reduced IgM and IgG3 serum concentrations [20]. TI responses to a subset of antigens is abrogated in these mice whereas TD responses are generally normal. AID is expressed in germinal center B cells and is an essential component for both CSR and somatic hypermutation. Consequently, AID deficiency results in a complete defect in class switching leading to a hyper-IgM phenotype [21, 22]. CD40L is expressed on activated T cells and it has a crucial role in B cell affinity maturation and isotype switching in germinal centers. Deficiency of CD40L leads to abrogated TD antigen-specific humoral response (only IgM production), whereas TI antigens are still able to initiate antigen-specific Ig production of all isotypes except IgE [5].

Moreover, different studies suggested a possible functional involvement of FLCs in different autoimmune disorders, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and multiple sclerosis [23-26]. Here, we additionally analyzed the production of FLCs in different preclinical models associated with autoimmunity.

Materials and Methods

Mice

Btk-, AID-, CD40L-, and FcγRIIb- deficient, and CD19-hBtk^{WT}, MHCII-hBtk^{WT}, Bcl-2, MRL/Lpr transgenic (Tg) mice have been described in previous studies [5, 20, 22, 27-32]. All mice, including wild-type C57BL/6 control mice (Harlan Laboratories, The Netherlands), were bred and maintained under specific pathogen-free conditions. Experimental protocols were reviewed and approved by the Erasmus MC Committee of animal experiments. Serum samples from all mice were analyzed at the age of 10–40 wk, n=3 per group.

Analysis of FLC in serum

Serum samples were precipitated to deplete high amounts of albumin by using trichloroacetic acid/acetone as previously described [33]. Subsequently, samples were separated by SDS-PAGE and blotted overnight onto a PVDF membrane (Bio-Rad Laboratories, Veenendaal, The Netherlands). Then, membranes were washed with PBS/0.1% Tween-20 for 5 minutes, blocked in 2% nonfat dry milk in PBS/0.1% Tween-20 (block buffer) for 1 hour, and incubated with 0.1 µg/mL HRP-labeled goat anti-mouse κ light chain (SouthernBiotech, Birmingham, Ala) diluted in block buffer for 1 hour. After washing the membranes with PBS/0.1% Tween-20 3×10 minutes, ECL (GE Healthcare, Eindhoven, The Netherlands) was used for detection of immobilized antibodies according to the manufacturer's protocol, followed by film (CL-XPosure film; Thermo Scientific, Etten-Leur, The Netherlands) exposure and development. Densitometry was performed on a GS-710 Calibrated Imaging Densitometer using software package Quantity One (Bio-Rad Laboratories) to analyze the density of the appropriate bands.

Isolation of hapten-specific FLC monomers and dimers

Trinitrophenol (TNP)-specific IgG (1B7-11, ATCC) (American Type Culture Collection, Manassas, Virginia) was purified with protein G-sepharose (GE Healthcare), reduced and alkylated to separate heavy and light chains. Proteins were eluted with 6M guanidine and subsequently loaded on to a gel filtration column (HiLoad 16/60 Superdex 200 pg, GE Healthcare) to separate heavy chains and FLCs. Subsequently, samples were dialyzed versus PBS, concentrated, eluted with 6M guanidine and loaded on to a gel filtration column once more to separate monomeric and dimeric FLCs. Finally, samples were dialyzed against PBS and concentrated again, and tested for the presence of FLC monomers and dimers and antigen-specific binding capacity.

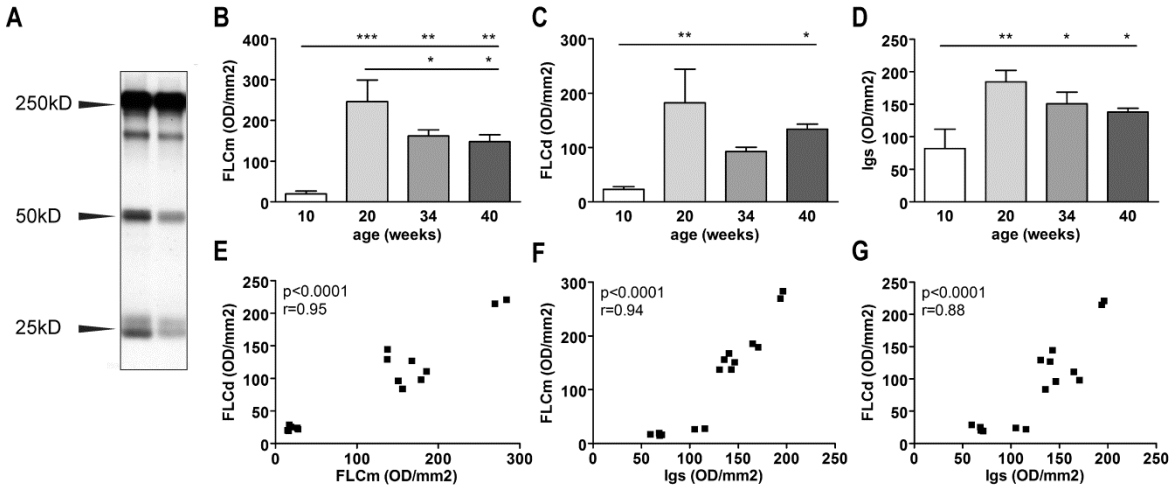

Figure 1. Serum FLC monomer and dimer and immunoglobulin concentrations highly increase in C57Bl/6 mice between 10 and 20 weeks of age. A, Serum from C57Bl/6 mice was analyzed for the presence of light chains by using western blot; FLC monomers (25kD), FLC dimers (50kD) and immunoglobulins (250kD; most sensitive at this position). A significant increase in expression of FLC monomers (**B**), dimers (**C**) and immunoglobulins (Igs; **C**) was detected between 10 and 20 weeks of age. This increase was still present after 34 and 40 weeks, although a slight decrease was seen compared to 20 weeks. There was a clear correlation between FLC monomer and dimer (**E**), FLC monomer and immunoglobulin (**F**), and FLC dimer and immunoglobulin (**G**) expression. For this latter analysis all age-groups were combined. * = P<0.05, ** = P<0.01, *** = P<0.001. FLC: immunoglobulin free light chain; OD: optical density.

Assessment of antigen-specific binding of FLCs

8 mg/mL ovalbumin (OVA) and 10 mg/mL bovine serum albumin (BSA) were conjugated with 5 and 1mM TNP, respectively, by 2 hours incubation at room temperature followed by 3 subsequent dialysis steps against PBS. High binding plates were coated o/n (4^{o}C) with TNP-OVA or TNP-BSA (50 µg/mL). Subsequently, plates were blocked with 1% BSA/0.05% Tween-20/PBS for 1 hour (RT) and incubated with either monomeric or dimeric TNP-specific FLC fractions diluted in 0.05% BSA/0.1% Tween-20/PBS (assay buffer) at different concentrations for 1 hour (RT). After incubation, plates were incubated with 0.1 µg/mL HRP-labeled goat anti-mouse κ light chain (SouthernBiotech) diluted in assay buffer for 1 hour (RT). Finally, TMB was used as a substrate and the enzymatic reaction was terminated by adding 0.9 M H_2SO_4. Between incubation steps, wells were washed three times with PBS-T (0.05% tween-20).

In addition, OVA-TNP was run on gel, and blotted overnight onto PVDF membrane, which was then blocked in 5% nonfat dry milk. Subsequently, membranes were washed with PBS/0.1% Tween-20 for 5 minutes and incubated with either 2.5µg monomeric or dimeric TNP-specific FLC fractions diluted in 5% BSA/0.1% Tween-20/PBS for 1.5 hours. After washing the membranes with PBS/0.1% Tween-20 3×10 minutes, antigen-specific FLC-

binding was detected by using 0.05 µg/mL HRP-labeled goat anti-mouse κ light chain. ECL prime (GE Healthcare) was used for detection of immobilized antibodies after a final wash step.

Statistical analysis

Differences in the amount of FLC (indicated by density of the bands on western blot) present in serum from mice of different age groups were analyzed by using a One-Way ANOVA and a subsequent Bonferroni's Multiple Comparison Test. Correlations between the amount of FLC monomers, dimers, and total immunoglobulins per serum sample were determined by the Pearson or Spearman correlation. P-values <0.05 were considered statistically significant. Based on the results from binding experiments by ELISA binding affinity was calculated by using Lineweaver-Burke and Eadie-Hofstee plots. All analyses were performed by using GraphPad Prism, version 5.03.

Results

Total FLC and immunoglobulin serum concentrations increase with increasing age in wild-type mice

Presence of FLCm, FLCd, and Igs in serum from wild-type C57BL/6 mice was analyzed by western blotting using an anti-mouse kappa light chain antibody (Figure 1A). Presence of FLCm and FLCd and Igs significantly changed during aging (FLCm: $P<0.0001$, FLCd: $P=0.002$, Igs: $P=0.001$; One-Way ANOVA) (Figure 1B-D). Little staining was detected for FLCm, FLCd, and Igs in mice 10 weeks of age, but their presence was highly increased at 20 weeks of age (FLCm: $P<0.001$, FLCd: $P<0.01$, Igs: $P<0.01$; Bonferroni test). At 34 (FLCm: $P<0.01$, Igs: $P<0.05$; Bonferroni test) and 40 weeks (FLCm: $P<0.01$, FLCd: $P<0.05$, Igs: $P<0.05$; Bonferroni test) of age, FLC and Ig presence was still increased as compared to 10 week old mice. After 20 weeks however, there appeared to be a slight decrease in all parameters, though this was only significant for FLC monomers (Figure 1B). Expression of serum FLCm, FLCd, and Ig all highly correlated with each other (Figure 1E-G), suggesting a balanced production of all 3 parameters during ageing.

Production of FLCm and FLCd and FLCd/FLCm ratio changes are highly different between wild-type mice and AID- and Btk KO mice

To delineate whether FLC production is dependent on isotype class switching, the presence FLCm, FLCd and Igs in serum was compared between wild-type mice and Btk- (deficient for

IgM and IgG3), AID- (only IgM production), and CD40L-deficient mice (high IgM, low IgG/IgA production). All three knockout (KO) strains showed a decreased production of FLCm, FLCd,

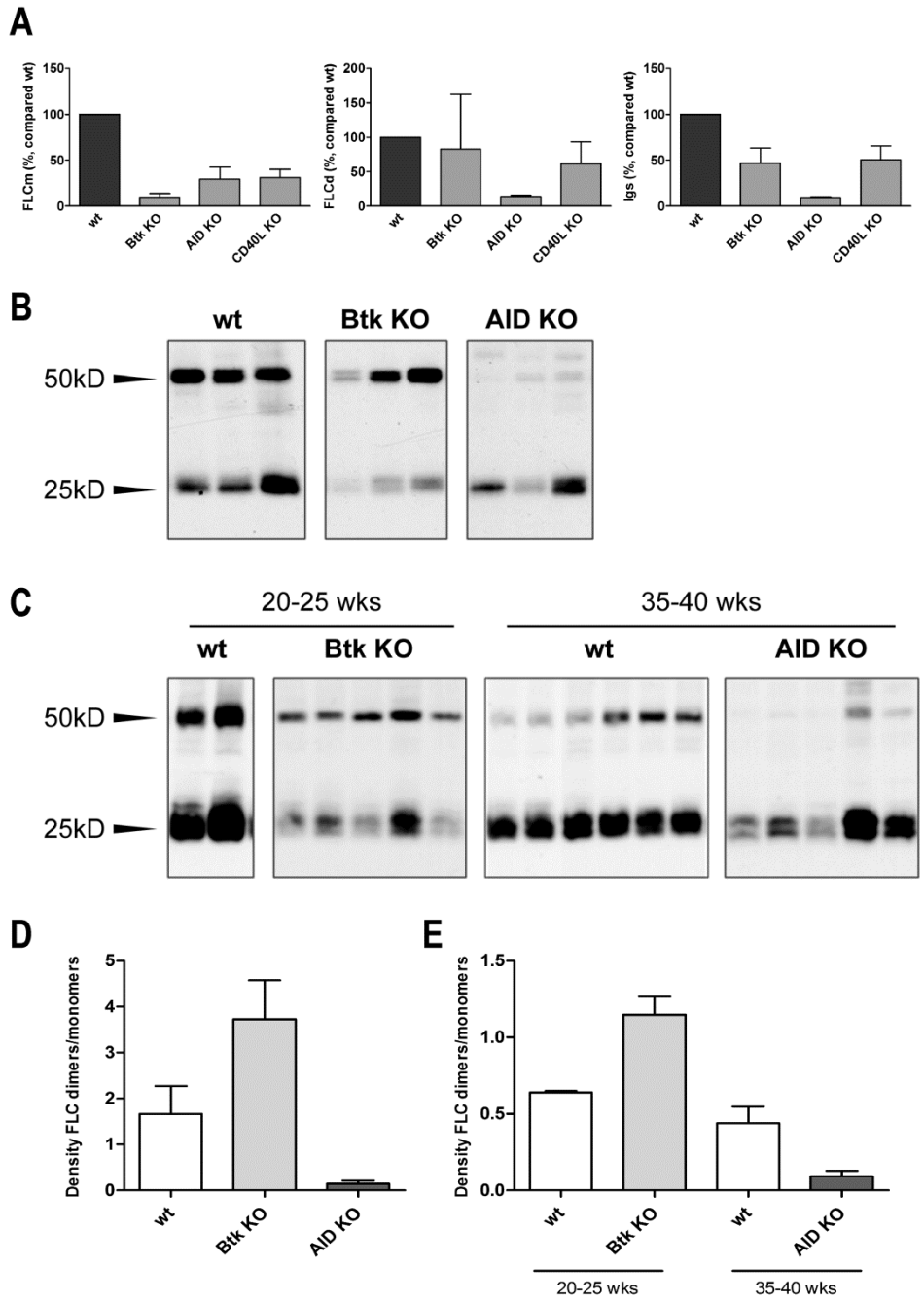

Figure 2. Serum FLC monomer and dimer and immunoglobulin production highly differs between wild-type and Btk- and AID knockout mice. Serum from 10 week old mice (13 weeks for CD40L KO mice and age-matched controls) was analyzed for the presence of FLC monomers and dimers, and immunoglobulins by using western blot (**A**). Interestingly, AID KO mice showed an almost complete absence of FLC dimer expression, in contrast to Btk KO mice, which showed a highly decreased FLC monomer production (Blots shown in **B**). This discrepancy in FLC monomer/dimer expression is independent on age since 20-25 (**C**, left), and 35-40 (**C**, right) week old mice show similar FLC production patterns. Ratios between FLC dimers and FLC monomer expression are shown in **D** (blots: figure B) and **E** (blots: figure C). Igs: immunoglobulins; wt: wild-type; FLC: immunoglobulin free light chain; KO: knockout.

and Igs (Figure 2A). Most prominent was the decreased FLCm production in Btk KO mice, in contrast to the marked decrease in FLCd in AID KO mice. In serum of Btk KO mice of 10 weeks of age, FLCm was hardly detectable whereas FLCd were clearly present. In contrast, 10 weeks old AID KO mice only produced FLCm (Figure 2A and B). Older mice showed comparable production patterns of FLCs, although FLCm appeared to be moderately present in Btk KO mice and minimal FLCd could be detected in 2 out of 5 AID KO mice (Figure 2C). Ratios of FLCd production versus FLCm production per mouse are shown in Figures 2D and E. Data of CD40L KO mice are not shown in figure 2B-E due to a difference in age compared to the other mice tested. Although FLC and Ig production was somewhat lower compared to age-matched controls, FLCm and FLCd were both clearly produced in CD40L KO mice. Serum from wild-type mice always contained both FLCm and FLCd (Figure 2B and C).

Increased FLC production in mice strain prone to develop autoimmunity

CD19-hBtk^{WT}, MHCII-hBtk^{WT}, Bcl-2, and MRL/Lpr transgenic (Tg) mice all show an increased survival of B cells and a reduced apoptosis of autoreactive B cells resulting in enhanced production of (auto)antibodies (Kil, Hendriks *et al*. submitted, and [29, 30]). FcγRIIb deficiency results in the absence of a negative feedback mechanism on activated B cells also resulting in enhanced antibody production [31]. These mouse strains were tested because of their association with autoimmunity and all showed an enhanced secretion of FLCm, FLCd, and Igs, although this increase was minimal in CD19-hBtk^{WT} mice (Figure 3). Interestingly, FcγRIIb KO mice clearly showed a highly increased production of both FLCm and FLCd as compared to wild-type mice, but the ratio between both types significantly changed since FLCd production was less increased than FLCm production (Figure 3).

Absence of an equilibrium balance between FLCm and FLCd

Based on the findings of FLCm and FLCd production in Btk KO and AID KO mice it appears that FLCm do not combine to FLCd and that FLCd do not split into FLCm *in vivo*. To verify this *in vitro*, we reduced and alkylated TNP-specific IgG, isolated TNP-specific FLCs, and separated these by gel filtration. FLCm and FLCd clearly showed 2 distinct peaks during the second gel filtration step which were collected separately (Figure 4A). An early elution fraction was detected as a shoulder left from the FLCd peak, indicating the presence of some polymeric FLCs. After subsequent dialysis steps against PBS, no FLCd was present in the FLCm fraction. In the FLCd fraction also FLCm was present, which may indicate that FLC dimers were split in monomers after isolation and dialysis (Figure 4B).

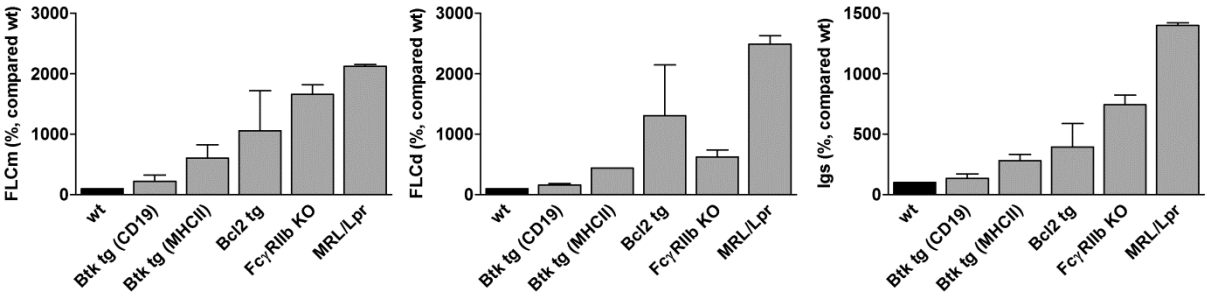

Figure 3. Serum FLC monomer and dimer expression highly differs between wild-type mice and mice associated with autoimmunity. Serum from 10 week old mice was analyzed for the presence of FLC monomers and dimers by using western blot. Transgenic mouse strains associated with autoimmunity all showed an increased expression of FLC monomers and dimers, and Igs (minimal for Btk tg (CD19)), as compared to wild-type mice. FLCm/d: immunoglobulin free light chain monomer/dimer; Igs: immunoglobulins; OD: optical density; KO: knockout; tg: transgene.

FLCd bind antigen with higher affinity than FLCm

The fact that FLCm and FLCd appear to be secreted as separate isoforms might imply different functions for both entities. Binding affinity of the FLCm and FLCd fractions isolated from TNP-specific IgG was analyzed by an antigen-specific ELISA and western blotting. Using an ELISA procedure, FLCm hardly showed antigen-specific binding to TNP-BSA whereas moderate binding was detected on OVA-TNP. In contrast, a clear binding was observed of FLCd to both TNP-BSA and TNP-OVA (Figure 5A). Quantification of the binding affinity in the ELISA procedure using TNP-OVA by using Lineweaver-Burke and Eadie-Hofstee plots indicated a 12-fold higher binding affinity of FLCd compared to FLCm (Lineweaver-Burke analysis: FLCm: 0.64 μM; FLCd: 0.052 μM).

Using western blotting, a similar difference in antigen-specific binding capacity to TNP-OVA was found between FLCm and FLCd when equal amounts of FLC were incubated (Figure 5B).

Discussion

In this study, we demonstrate that plasma cells are able to differentially produce FLC monomers or FLC dimers. Based on the preferential production of different immunoglobulin subclasses by Btk KO and AID KO mice, FLCm production is found associated with IgM production, in contrast to FLCd which are predominantly produced in parallel with IgG1. The production of FLCd may be of functional importance, because binding studies show that dimers have better antigen-specific binding capacities compared to FLCm. In addition to previous findings in human autoimmune disorders, we also observed a highly increased FLC production in mouse strains associated with autoimmunity which was proportional to the increase in Igs.

In accordance with previous findings in humans, low serum FLC concentrations were observed in young mice which increased during life [34-37], while both FLCm and FLCd are clearly present. Similar findings were observed for Igs, and the clear correlations between FLCs and Igs suggest that during ageing a fixed fraction of light chains is secreted as FLCm and FLCd. To date, little is known about regulatory mechanisms involved in FLC production other than the influence of the B cell activation state [12, 15, 16]. In this study we investigated whether FLC production is dependent on the immunoglobulin subclass that is being produced by analyzing Btk-, AID-, and CD40L KO mice. Clear differences in the production of monomeric and dimeric forms of FLC were found between wild-type, Btk KO (deficient for IgM and IgG3), and AID KO mice (only IgM production). Based on the clear differences between both KO strains in their Ig isotype serum profile, these data suggest that FLCm production may be associated with IgM production, whereas FLCd may be produced after

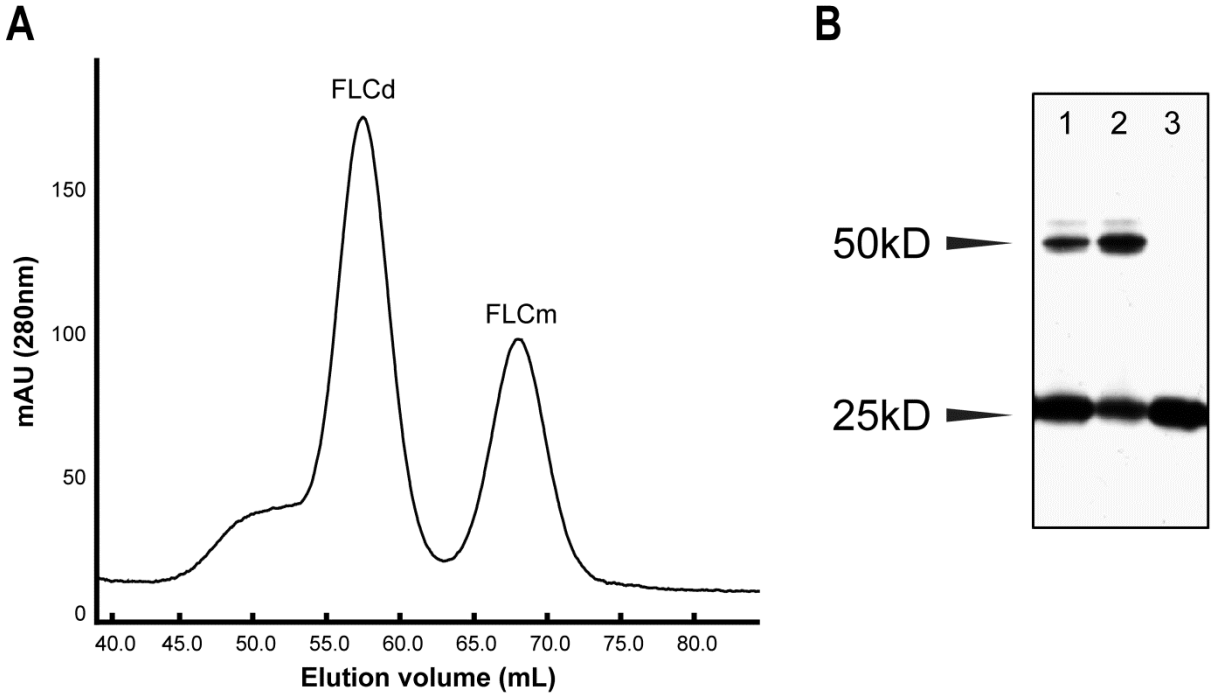

Figure 4. Separation and isolation of TNP-specific FLC monomers and dimers by gel filtration. TNP-specific mouse IgG was reduced and alkylated first and subsequently subjected to 2 cycles of gel filtration: first filtration to separate heavy and light chains, second filtration to separate FLC monomers (FLCm) and dimers (FLCd). **A**, A mixture of FLCm and FLCd was applied on a HiLoad 16/60 Superdex 200 pg gel filtration column running at 1 mL/min at room temperature with 6M guanidine hydrochloride buffer. Two clear peaks were detected and isolated separately. **B**, Western blot analysis of the FLC fraction before the second gel filtration shown in **A** (lane 1), and FLCd (lane 2) and FLCm (lane 3) fractions isolated during the second gel filtration step. Equal amounts of FLCs (6ng) were loaded on gel. Lane 2 shows an increase in FLCd compared to lane 1, but FLCm can still be detected. Lane 3 clearly only shows the single presence of FLCm. mAU: milliabsorbance units.

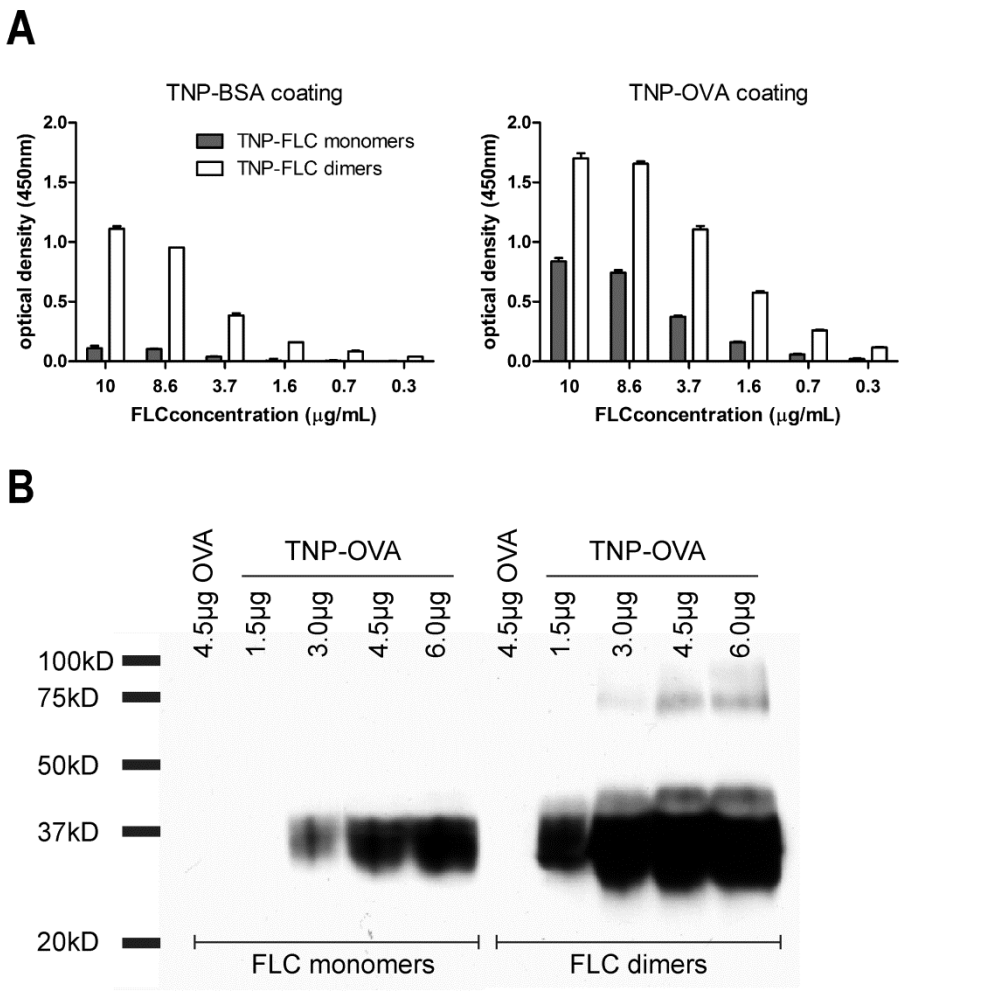

Figure 5. TNP-specific FLCd bind antigen with higher affinity than TNP-specific FLCm. A, Binding analysis of TNP-specific FLCm and FLCd to TNP-OVA and TNP-BSA (50 µg/mL) coated high binding plates demonstrates that FLCd bind antigen significantly better than FLCm. Quantification of binding affinity to TNP-OVA by using Lineweaver-Burke plots indicated binding affinities of 0.64 µM and 0.052 µM for FLCm and FLCd, respectively. **B**, TNP-OVA was run on gel, and blotted on PVDF membrane. Binding of 2.5µg monomeric (left blot) or dimeric (right blot) TNP-specific FLC was subsequently analyzed. Higher binding affinity of FLCd compared to FLCm is clearly demonstrated. TNP: trinitrophenol; FLC: immunoglobulin free light chain.

class switching to IgG1. The fact that CD40L KO mice do not show a FLC phenotype that resembles that of AID KO mice might be explained by the fact that CD40L KO mice are still able to produce all Ig isotypes except IgE following a TI antigen challenge. Whether thisdifference in FLC secretion is really isotype-specific needs further study. Analysis of Ig isotypes in all sera tested in this study is therefore essential and currently in progress. Furthermore, analysis of FLC isoforms in human subjects having disorders that are associated to an increased production of a specific Ig-subclass could be indicative for human monomer and dimer production. Interesting disorders to explore would be the hyper-IgM syndromes HIGM2, which is caused by AID deficiency and is a human counterpart of the

AID KO mouse model [38], and HIGM1, which is caused by a CD40L deficiency and is also characterized by high IgM expression and a virtual absence of other Ig isotypes [39].

FLCd are formed by covalent or noncovalent binding of two FLCm. Structural reasons for the differences in monomer interaction and the formation of FLCd are complex and not clearly understood [40]. Nevertheless, it is generally believed that there may be a balance between FLCm and FLCd. In one study, a significant variance in dimerization constants (K_D) was found between different light chains, which was shown to be attributable to the presence of a specific residue at position 96. At this position an aromatic or hydrophobic residue enhanced dimer formation, whereas a charged residue favored FLCm formation [41]. The results of the current study do not support the presence of an equilibrium balance between FLCm and FLCd, since either FLCm or FLCd is almost exclusively found in AID or Btk KO mice, respectively. Furthermore, we showed that isolated antigen-specific FLCm did not form FLCd after prolonged incubation in PBS. With respect to FLCd it was found that some FLCm were present after separation by SDS-PAGE. However a clear FLCd peak was observed during isolation via gel filtration, which may indicate that some FLCd may be split during the experimental isolation conditions, which is subject of present research. Furthermore, analysis of structural differences between FLCm and FLCd may be due to different amino acid sequences of light chains found in FLCm versus FLCd.

The fact that FLCm and FLCd appear to be distinct entities suggests that they may have different functional characteristics as well. This is supported by the *in vitro* binding data, which show that FLCd have a significantly higher antigen-specific binding affinity compared to FLCm. Increased binding affinity of FLCd can likely be explained by the presence of two variable regions that contribute to antigen-binding, compared to one variable region in FLCm. These differences in binding affinity must be considered in future experiments. So far, studies analyzing the biological functions of FLCs, including passive FLC sensitization models described by our group [17, 42, 43], did not discriminate between FLCm and FLCd.

Finally, we investigated FLC expression in mouse strains associated with autoimmune disorders like RA (FcγRIIb KO [44]) and SLE (Btk tg, Bcl-2, MRL/Lpr; Kil and Hendriks *et al*. submitted, and [29, 30]). In line with findings in human subjects, these preclinical models also showed highly increased serum FLC and Ig concentrations, with the exception of CD19-hBtk^{WT} mice. In line with earlier observations, CD19-hBtk^{WT} mice showed comparable Ig production with wild-type mice which was associated with a comparable FLCm and FLCd production. MHCII-hBtk^{WT} mice did show an increased Ig production in contrast to earlier findings [28, 45], but this was also accompanied with an increased FLCm and FLCd production. In none of the strains we observed independent induction of FLC production.

In conclusion, we provide evidence for a distinct production of FLCm and FLCd which seems to be dependent on the immunoglobulin subclass that is being produced. The single presence of FLCm or FLCd in serum or in *in vitro* conditions is indicative for the absence of an equilibrium between both isoforms. Moreover, FLCd showed to have a significantly higher antigen-specific binding affinity compared to FLCm suggesting a putative more dominant role in mediating antigen-specific immune responses. Future research should address these functional differences in *in vivo* and *in vitro* models and differences between monomeric and dimeric FLC expression requires more attention in different human disorders.

Acknowledgements

We would like to thank Marjolein de Bruijn (Erasmus MC, Rotterdam, The Netherlands) for collecting blood from all mice.

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11

General discussion

Part of this chapter is published in *Current Pharmaceutical Design*, 2011

Immunobiology of antigen-specific immunoglobulin free light chain in chronic inflammatory diseases

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The finding that FLCs are capable of mediating antigen-specific mast cell activation creates a new look at disorders in which B cells and mast cells are involved. In this respect, allergic diseases are of great interest. Allergy is an exaggerated immune reaction to usually harmless foreign proteins present in food and airborne pollens for example. Allergic hypersensitivity can be subdivided to IgE- and non-IgE-mediated allergies [1]. Allergic responses via IgE are intensively investigated since IgE is thought to play an important role in many allergic diseases [2-4]. IgE can bind antigen with high affinity and crosslinking of FcεRI-bound IgE on mast cells by multivalent antigen causes mast cell activation leading to the release of multiple mediators via exocytosis of the characteristic granules containing mediators such as histamine, 5-HT and tryptase along with cytoplasmic stored and newly generated pro-inflammatory mediators including cytokines, and chemokines and certain growth factors [5-7]. Together these mediators mediate a local inflammatory response leading to an early and subsequent late phase response. Despite this important contribution of IgE to many allergies, a significant proportion of patients (up to 40%) suffers from a non-IgE-mediated allergy as described in **chapter 2**. Examples of disorders displaying such a high percentage of patients in which IgE cannot be identified as the critical regulator of inflammation are food allergy, rhinitis, asthma, and dermatitis [8]. Nevertheless, mast cells still appear to play a key role in mediating these disorders. Despite some differences, many clinical and immunological similarities are recognized between IgE and non-IgE-mediated allergies and treatment of each with corticosteroids is equally effective. The question arises what mechanism(s) are responsible for causing the various mast cell-mediated and allergen-specific, but non-IgE-mediated, allergic diseases. What mechanism(s) are responsible for causing the various mast cell-mediated responses is an essential question that also applies to other inflammatory disorders in which mast cell activation appears to play a prominent disease modifying role, including rheumatoid arthritis (RA) and cancer. An antigen-specific mediated pathology is indicated in RA since a substantial number of RA patients displays antigen-specific antibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) [9]. In cancer this antigen-specific immune activation is less well described although, in contrast to allergy and RA, antigen-specific IgE might be involved in protective, anti-tumoural responses [10]. In contrast, mast cell activation by other factors can lead to tumour progression (**chapter 7**).

Besides IgE, several other factors can induce mast cell activation. These include IgG via FcγRI or FcγRIII (in mice), complement components like C5a via C5aR, cytokines, toll-like receptor ligands, bacteria, viruses, and via interaction with T cells [11-14]. Moreover, as extensively described in this thesis, FLCs can mediate antigen-specific mast cell activation. A number of studies has indicated a possible functional role of (antigen-specific) FLCs in

preclinical models of cow's milk allergy, asthma, and inflammatory bowel disease (IBD) which are not described in this thesis. In addition, in this thesis we showed in a preclinical mouse model that FLCs can be involved in tumour progression (**chapter 8**). Moreover, this thesis describes several studies on different human disorders (rhinitis, chronic rhinosinusitis, idiopathic pulmonary fibrosis and hypersensitivity pneumonitis, rheumatoid arthritis, and cancer), indicating a possible disease-modifying role for FLCs in human as well. This is probably not only via its action on mast cells, since it was shown that FLCs also bind to basophils, and its crosslinking leads to enhanced basophil activation (**chapter 9**). All clinical and preclinical data on FLCs described in this thesis will be discussed below and related to previous and more recent findings concerning FLCs. Finally, these data will be integrated in a schematic overview of current concepts on the role of FLCs in pathologic and physiologic immune responses in human.

Upper and lower airway disorders

Asthma and rhinitis are common co-morbidities, suggesting a concept of "one airway, one disease" [15, 16]. Allergy of the upper airways manifests as rhinitis and is characterized by inflammation of the nasal mucosa. Rhinitis can be classified into infectious, atopic (IgE-mediated), and non-atopic/non-infectious, with this latter group being highly heterogeneous but yet comprising a substantial number of rhinitis patients [8]. A subpopulation of patients with non-atopic rhinitis shows nasal mucosal inflammatory characteristics comparable to atopic rhinitis, including the increased number of mast cells [17-21]. However, a role for IgE in these disorders cannot completely be excluded since the local production of IgE at the site of inflammation is demonstrated [22-25]. To delineate a putative contribution of FLCs to the mast cell-mediated effects in these patients, FLC presence and tissue localization was analyzed in a group of patients with a non-atopic persistent rhinitis (non-allergic rhinitis with eosinophilic syndrome (NARES)) as described in **chapter 3**. The presence of tryptase and eosinophilic cationic protein in nasal secretions from both IgE-mediated rhinitis and NARES patients clearly showed the involvement of mast cells and eosinophils in both disorders. Systemic and local IgE concentrations however, were at the level of healthy controls in NARES patients and no specific IgE to seasonal or perennial allergens was detected in this group. IgE-mediated rhinitis patients showed high antigen-specific IgE concentrations in serum and nasal secretions as expected. Interestingly, local FLC concentrations were increased in both IgE-mediated rhinitis and NARES patients compared to control subjects, but this increase was significantly higher in the NARES group. Only a slight but significant increase of FLCs was detected in the serum from NARES patients, which is suggestive for a local immune response. Furthermore, higher numbers of FLC positive cells were detected within the nasal

mucosa of both allergic groups. Laser microdissection and RNA analysis demonstrated that mast cells were amongst the FLC-positive cells suggesting that FLC-mediated mast cell activation might be a mechanism involved in non-IgE-mediated rhinitis.

Asthma is a relatively common chronic disorder of the lower airways and related to rhinitis. Previously, it was shown that FLCs are also involved in asthma pathology using a murine experimental model showing clear similarities with adult asthma in humans [26]. In this model, topical cutaneous sensitization with DNFB followed by intranasal exposure to cognate antigen leads to a mast cell-dependent pulmonary hypersensitivity reaction [27]. F991 treatment was able to completely block the early phase hypersensitivity responses as indicated by a reduced mast cell activation and bronchoconstriction. Late phase responses, manifested by mucosal leakage, neutrophilic cellular infiltration in BAL fluid and tracheal hyperreactivity 24 and 48 hours after challenge were also attenuated after F991 treatment. In addition, passive sensitization with TNP-specific FLCs induced comparable early phase responses as compared with the active model. This response was also abrogated after F991 treatment and was shown to be mast cell dependent. Analysis of serum from atopic and non-atopic asthma patients indicated that kappa FLC concentrations are higher in both groups compared to non-asthmatic age-matched controls [26], which is in line with the small but significant increases in serum FLC concentrations in non-atopic rhinitis patients described in **chapter 3**.

Another disorder of the upper airways is chronic rhinosinusitis (CRS) and was subject of research in **chapter 4**. CRS is a chronic inflammatory disorder of the nose and paranasal cavities and is divided in two different entities based on histology and inflammatory patterns: CRS with (CRSwNP) and without nasal polyps (CRSsNP). CRSwNP in Caucasian patients is characterized by a Th2-mediated eosinophilic immune response with high IL-5, ECP and IgE concentrations, whereas CRSsNP is mainly a Th1 immune response with high levels of IFN-γ and TGF-β [28-31]. Despite these differences in underlying disease mechanisms, FLCs were found to be increased in both disease types although CRSwNP patients showed highest FLC concentrations. In line with our findings in rhinitis patients (**chapter 3**), FLC concentrations were only locally increased. In contrast however, this increase was most prominent in a subgroup of patients showing evidence for the involvement of IgE. Specific IgE has been identified in nasal tissue from patient with nasal polyposis [32, 33] and also with non-IgE-mediated rhinitis [34-36]. Evidence for the functional involvement of IgE in polyposis was obtained by *ex vivo* crosslinking of IgE in polyp tissue, resulting in an increased release of early phase mast cell mediators in polyposis tissue compared to control tissue [37].

Interestingly, a decrease in local FLC concentrations was observed in polyposis patients treated with anti-IL-5 whereas systemic FLC concentrations were unaffected. Since anti-IL-5 treatment is effective in reducing nasal polyp scores (Gevaert, 2011, J Allergy Clin Immunol. *in press*), it is tempting to speculate on a functional role for FLCs in mediating this disease. However, the absence of a correlation between a decrease in FLC concentrations and decrease in polyp score does not directly support this hypothesis (**chapter 4**). In addition, the decrease in FLCs production after anti-IL-5 could also indicate a role for IL-5 in steering immunoglobulin secretion towards more prominent FLCs secretion. Mechanisms underlying this FLC production and secretion are poorly understood and briefly discussed below.

A third group of airway disorders analyzed in this thesis are interstitial lung diseases (ILD), comprising a diverse group of disorders affecting the lung parenchyma that are classified together because they share similar clinical, radiographic, and physiologic features [38]. Two frequent and complex ILD are idiopathic pulmonary fibrosis (IPF) and hypersensitivity pneumonitis (HP) which are analyzed for the presence of FLCs in **chapter 5**. Similar to the findings described thus far for rhinitis and asthma, both systemic and local FLC concentrations were increased in both disease types. However, compared to controls systemic concentrations in ILD patients appeared to be significantly higher than those observed in asthma and rhinitis patients. Possibly this can be attributed to a bigger tissue area affected by the disease. Nevertheless, the largest differences were again observed at local sites (in the BAL fluid) in the majority of patients, and local FLC production in HP and IPF patients is suggested by the high presence of local B cells and plasma cells. Despite the absence of measurable amounts of tryptase in the BAL fluid of HP and IPF patients, clear mast cell activation was demonstrated in both disease types by immunohistochemistry. The involvement of IgE in IPF and HP is not likely, because IgE concentrations in BAL fluid are low and not increased compared to healthy controls. Antigen-specific IgG might be responsible for mast cell activation in HP patients. Increased IgG concentrations in BAL fluid of these patients are shown in **chapter 5**, and specific IgG against the sensitizing antigen have been demonstrated in HP lungs [39]. Thus, FLCs and IgG together might provide a mechanism by which antigen-specific mast cell activation takes place in the lungs of HP patients. In contrast, IgG concentrations were also low in IPF patients, and IPF is not considered to be an antigen-specific mediated disease. Instead, viral infections seem to be common in these patients [40, 41] and although IPF is a multi-factorial disease, a growing body of evidence implicates viruses as co-factors, either as initiating or exacerbating agents [42]. Viral infections increase the occurrence of FLCs and interestingly, we have recently shown that FLC concentrations are greatly increased during viral myocarditis in mice and that FLCs may play a protective role in the pathogenesis of this disease [43]. This latter observation

impedes a clear association of FLC to either progression or suppression of IPF, which is further hampered by the lack of good IPF disease models. Extensive analysis of mast cell activation in lung tissue and FLC concentrations in BAL fluid or lung tissue of the same patients might give more insight in this question.

Autoimmune diseases; rheumatoid arthritis

In contrast to the allergic type disorders described above, rheumatoid arthritis (RA) is a systemic autoimmune disorder featuring inflammation of synovial tissues mainly considered as a T cell and macrophage-dependent disease. Besides, autoantibody-producing B lymphocytes play a pivotal role in RA pathology which is greatly supported by effective treatment with B cell depletion therapy using rituximab [44-46]. Additionally, critical disease regulatory functions have also been ascribed to mast cells and mast cell mediators in RA disease pathology [47-50]. Increased serum FLC concentrations in RA patients are shown to correlate with disease activity (**Chapter 6** and [51, 52]), suggesting that FLCs may be involved in mediating synovial mast cell activation. Similar to these findings, high FLC concentrations in multiple sclerosis patients correlated with an increased disease severity and clinical relapse in systemic lupus erythematosus (SLE) patients was correlated to a preceded rise in urinary FLCs [53, 54].

Comparable to rhinitis, CRS, HP and IPF patients (**chapters 3, 4, and 5**), the highest FLC concentrations that are detectable in RA patients are at the site of active inflammation, i.e. in the synovial fluid and tissue of affected joints. In RA patients, extremely high synovial fluid FLC concentrations were detected frequently exceeding those measured in serum. Probably as a consequence of this, in RA patients serum FLC concentrations reflect spill-over of FLC from local production in the inflamed joints [55]. Again, local production is further supported by the presence of B cells and plasma cells within synovial tissue of affected joints. Rituximab treatment in a cohort of 50 RA patients with active disease showed that FLC serum concentrations decreased after the start of treatment and more strikingly, relative changes in FLC concentrations correlated with changes in disease activity score and systemic markers of inflammation. Since anti-TNF treatment did not significantly change serum FLC concentrations although being clinically effective, changes in FLC concentrations are not a general feature of clinical response to treatment. This implies that the effect of rituximab may partly rely on reduction of FLC production by pathogenic B cells, or could be due to effects on antigen presenting capacity of the local B cells.

Another autoimmune disorder associated with possible involvement of FLCs is inflammatory bowel disease (IBD). IBD is characterized by chronic, spontaneously relapsing

inflammations of the gastrointestinal tract, and occurs in Crohn's disease or ulcerative colitis [56]. The precise mechanisms underlying the development of IBD are unknown but mast cells appear to be crucial by releasing mediators such as histamine and tryptase without a clear contribution of IgE [57, 58]. The contribution of FLCs in IBD was analyzed using a mast cell-dependent murine IBD model [59], in which FLCs were shown to be a crucial factor to develop colonic hyperreactivity symptoms. In line with the findings in RA patients (**chapter 6**), analysis of FLCs in IBD patients and control subjects revealed that FLC concentrations are systemically increased, and that FLC staining at the site of inflammation (affected colon and ileum tissues in this study) was highly positive in IBD patients [60].

Cow's milk allergy

As indicated above, allergies (especially non-IgE-mediated) are of great interest to investigate a functional role for FLCs in mediating disease pathology. Although not a subject of this thesis, a role for FLCs in mediating cow's milk allergy (CMA) is implicated in recent research and will be briefly discussed below. In a recently developed murine CMA model, sensitization with one of the two major protein constituents of cow's milk, casein and whey, resulted in allergic symptoms after challenge with the appropriate antigen and involved mast cell activation [61]. Interestingly, whereas whey immunization clearly showed IgE-mediated symptoms, casein induced a predominantly FLC-mediated allergy [62]. In another study it was shown that the whey-induced CMA, which is highly IgE dependent under normal conditions, can be switched to a FLC-mediated response after depletion of $CD25^+$ cells in the sensitization phase [63]. In this study, a single anti-CD25 treatment before whey-specific IgE was systemically detectable, completely abrogated the production of whey-specific IgE, but did not influence early phase responses. A striking finding was that the anti-CD25 treated group showed increased FLC concentrations (especially in MLN) and the early phase response could be blocked by antagonizing FLCs with F991. This shift from IgE towards FLC production was suggested to be a result of the deletion of whey-specific effector T cells which are responsible for the induction of class switching in B cells to IgE/IgG1 under normal conditions.

Mast cells and FLCs in tumour-associated inflammation

The inflammatory component in allergic and autoimmune disorders is highly evident and recognized for many years already. In addition, it is becoming apparent that the tumour microenvironment, containing many inflammatory cells, is a critical determinant of malignant transformation and tumour proliferation. As outlined in **chapter 7**, the role of mast cells in

this process can either be beneficial or detrimental to tumour development, which may depend on the stage of tumour development during which mast cells infiltrate, the communication with other cell types, or on the tumour microenvironment. The tumour promoting effects of mast cells are mainly mediated by secretion of pro-angiogenic factors and by tissue remodelling, enhancement of tumour cell proliferation, and immunosuppression. However, many other inflammatory cells infiltrate into the tumour microenvironment. These cells, including tumour-associated macrophages, are also thought to have important roles in tumourigenesis [64-67]. Since mast cells are capable of affecting chemotaxis, proliferation, and activity of many of these cell types, they might be critical regulators of inflammation within the tumour microenvironment and consequently of tumour growth. Because of this mast cell involvement, the presence of FLCs in tumour tissue was analyzed in **chapter 8**. A combined analysis of both kappa and lambda FLCs demonstrated a profound FLC presence in tumour tissues of various aetiologies in multiple organs. Clear mast cell infiltration was also detected in essentially all tumour samples, supporting its prominent tumour infiltrating capacity as described above. To further characterize whether FLC expression was associated with the manifestation of clinical disease of cancer, kappa and lambda FLC expression was analyzed separately in breast cancer tissue from a large group of patients (**chapter 8**). Unexpectedly, mainly lambda FLC was shown to co-localize with tumour-infiltrating inflammatory cells, and this expression was highly associated with a poor clinical disease outcome. In contrast, this was not observed for kappa FLCs which staining predominantly co-localized to tumour cells. This discrepancy in kappa and lambda FLC expression within tissues was not observed in any other pathology studied before (**chapters 3-6**). Whether this consequently implies distinct functional capacities for both FLC isotypes is yet unknown but definitely warrants further research. The functional role of FLCs within tumour environment was demonstrated in **chapter 8** in a preclinical tumour model. Antagonizing FLCs within tumour tissue during tumour progression attenuated the growth significantly. Moreover, mast cell deficiency resulted in a tumour growth inhibition that was comparable to the growth after FLC blocking, suggesting that FLC-mediated mast cell activation results in a tumour environment that promotes tumour progression.

How FLCs influence tumour growth is still speculative, and raises several questions. Based on the data available so far, this response could be antigen-specific and results in mast cell degranulation [68]. However, this does not directly support a pro-tumourigenic role since mast cell degranulation is also suggested to be detrimental to tumours, in contrast to piecemeal degranulation [69]. Moreover, the similarities observed so far between FLC- and IgE-mediated responses in preclinical models together with the proposed involvement of antigen-specific IgE in protective, anti-tumoural immune responses [10], are in contrast to a tumour-promoting

function. Nevertheless, differences between the standard IgE-mediated anaphylactic exocytosis and mast cell degranulation mediated by TCF were demonstrated in earlier work (**chapter 1**). Therefore, FLC-mediated mast cell activation in the tumour environment might result in a more subtle release of mediators that promote tumour growth. On the other hand, the localization of mast cells appears to influence its effect on tumour growth. Peritumoural mast cells, the predominant phenotype observed in the tumour model described in **chapter 8**, are associated with promoting tumour growth, whereas intratumoural mast cells are associated with tumour growth suppression [70]. Finally, since FLCs are able to bind antigen it is of major interest to investigate target antigens within the tumour microenvironment that are potentially involved in FLC-mediated mast cell activation.

Basophil activation is enhanced by crosslinking of membrane bound FLC

The functional role of FLCs in mediating immune responses has mainly been focussed on its ability to induce mast cell activation and subsequent mediator release. FLC binding to other cell types is hardly investigated, except for neurons and neutrophils as described in **chapter 1**. Antigen-specific FLCs can bind to, and activate, murine dorsal root ganglion neurons upon application of appropriate antigen as measured by increases in intracellular Ca^{2+} [71]. Because of the well described neuro-immune interaction, this FLC-mediated neuron activation can influence immune responses including mast cell activation [72-74]. In addition, independent of their antigen-specificity, FLCs influence some essential neutrophil functions as it was demonstrated that FLCs significantly inhibited chemotactic neutrophil movement, reduced the activation of neutrophils, and attenuated neutrophil apoptosis [75, 76]. The binding of FLCs to basophils was demonstrated in **chapter 9** in four donors, all having some type of seasonal or perennial allergy. Interestingly, crosslinking of membrane-bound FLCs alone did not result in basophil activation, whereas a clear increase in activation was demonstrated in combination with IgE-mediated basophil activation. This is in line with recent findings showing that *in vitro* activation of mast cells by crosslinking of membrane-bound FLCs can only be demonstrated upon co-stimulation with IgE-crosslinking [77]. However, although all donors showed comparable expression of FLCs, this enhancement of IgE-mediated basophil activation was only demonstrated in two patients. What caused these inter-individual differences is currently unknown. Significant differences in IgE-mediated basophil activation are also demonstrated between different the donors, despite comparable membrane expression of IgE (**chapter 9**). Heterogeneity in basophil responses to IgE- and/or FLC-crosslinking possibly depends on intrinsic differences or additional modulatory signals influencing basophil function *in vivo* [78]. Inter-individual differences in human mast cell

functions and their responses to FLC-crosslinking should also be considered in future research.

The modulatory effect of FLCs on basophil function described in **chapter 9** implicates that research on the functional role FLC in preclinical disease models and human disorders should not be restricted to mast cell function alone. Basophils are increasingly being appreciated as critical inflammatory cells involved in allergy and immunoregulation [79, 80]. Moreover, some studies indicated an association between basophils and allergic asthma and rhinitis (FLC involvement described in **chapter 3**), and skin diseases, in which it was shown that basophil numbers increased during the late phase response upon allergen challenge [81-84]. Unfortunately, no marker genes other than for mast cells and plasma cells were included in the PCR analysis performed on RNA from isolated FLC-positive cells from nasal mucosal tissue of rhinitis patients (**chapter 3**). Current preliminary findings also demonstrated FLC-binding to human neutrophils which was highly patient-dependent (Braber *et al.*, submitted and unpublished data). IL-8 release after crosslinking of neutrophil-bound FLC was demonstrated but further functional aspects of this binding are lacking at this point and definitely requires extensive research in future experiments. Among other inflammatory cell types to be investigated are eosinophils as well, especially because of the high local FLC concentrations in NARES patients and the massive eosinophil infiltration of the nasal mucosa in these patients.

FLC production

Clear regulatory mechanisms involved in FLC production and secretion are largely unknown. As indicated in **chapter 1**, some studies demonstrated that immature B cells were responsible for FLC secretion, in contrast to mature B cells which showed a balanced production of heavy and light chains [85-87]. Specific B cell activating conditions that stimulate mature B cells or plasma cells to produce either FLCs or complete immunoglobulins (Igs) exclusively have not been described. In **chapter 10** it was attempted to find specific conditions that could lead to exclusive FLC or Ig production but these were not found. The studies described in this thesis do not reveal such specific conditions. This does not exclude the possibility of exclusive FLC production since only a limited number of specific conditions using knockout and transgenic mouse strains were tested. Moreover, FLC and Ig expression were analyzed in serum only. Since this reflects the FLC/Ig production within the whole body, effects on specific B cell subsets could be missed.

Based on all clinical data described in this thesis it is also hard to discover a clear pattern in FLC production in relation to the production of a specific Ig subclass. A combination of high

local FLC concentrations and IgE production was observed in atopic rhinitis and CRSwNP patients (**chapters 3 and 4**, respectively). However, high FLC concentrations were also found in the absence of local IgE in NARES, CRSsNP, HP, and IPF patients (**chapters 3, 4 and 5**, respectively). High FLC production was also observed in combination with high IgG and high IgG/IgM concentrations in HP and RA patients (**chapters 5 and 6**, respectively). In IPF patients we did not detect any decrease in either IgG or IgE (**chapter 5**). Together, this might support the earlier finding that immature B cells, which will be present at local inflammatory sites next to mature plasma cells, are responsible for the production of FLCs independent of the Ig subclass that will ultimately be secreted. This also is in line with the effects of $CD25^+$ cell depletion in the IgE-mediated whey allergy model described above [63]. This treatment resulted in an inhibition of whey-specific IgE production and a concomitant increased FLC production. As suggested in this study, anti-CD25 treatment possibly resulted in the deletion of whey-specific effector T cells which are responsible for maturation of B lymphocytes. Absence of these cells might result in the accumulation of immature B lymphocytes that predominantly secrete FLCs. Although highly speculative, the divergent disease mechanisms observed in the preclinical whey (IgE-mediated) and casein (FLC-mediated) allergy models [62], could imply that, compared to casein, whey induces a more pronounced antigen-specific T cell activation resulting in better B cell stimulation and subsequent maturation.

An unexpected observation described in **chapter 10** was the almost exclusive presence of monomeric FLCs in AID knockout mice and dimeric FLCs in Btk knockout mice. This was primarily striking because so far it was generally assumed that an equilibrium balance existed between FLC monomers and dimers. However, the findings in AID and Btk knockout mice indicate that this might not be the case. In addition, the fact that monomeric and dimeric mouse FLCs could be isolated separately and subsequent prolonged incubation in PBS did not result in the formation of dimeric FLCs out of monomeric FLCs (**chapter 10**) further supported this observation. Whether structural differences in light chains underlie the difference in phenotypic appearance observed remains unknown and needs further study. One study supporting a potential structural difference described a significant variance in dimerization constants (K_D) between different light chains which was shown to be attributable to the presence of a specific residue at position 96 (an aromatic or hydrophobic residue enhanced dimer formation, whereas a charged residue favored FLC monomer formation) [88].

The observation that FLCs can either be secreted as monomers or dimers raises two new questions in particular. What trigger causes B lymphocytes to produce monomeric or dimeric FLCs and is there a functional difference between both isoforms? Regarding the first

question, findings described in **chapter 10** suggest that the Ig subclass that will ultimately be produced determines which FLC isoforms will be secreted; i.e. FLC monomers in case of IgM production and FLC dimers in case of IgG production. A functional difference between both FLC isoforms is not directly addressed in this thesis. However, the significant difference in antigen-specific binding (about 12-fold difference) indicates a putative dominant role for FLC dimers in mediating antigen-specific immune responses. Comparing the early immune responses after passive immunization with either FLC monomers or dimers in a preclinical model for contact hypersensitivy for example (described in [68]), could give a first indication. As a consequence of this observed divergence, future research on human disorders might warrants a more detailed separate analysis of FLC monomer and dimer expression in case total FLC concentrations are increased.

Role of FLCs in tolerance and immune suppression

So far, the putative pro-inflammatory effects of FLC have been discussed in this thesis. However, several studies not described in this thesis indicated the involvement of FLCs in suppressing immune responses and will be briefly addressed in this paragraph. Similar to increased concentrations seen in patients with viral infections, FLCs were increased several days after initiation of a viral-induced experimental myocarditis model in mice [43]. Daily subcutaneous injection of polyclonal FLCs of unknown specificity starting one day before viral inoculation in this model resulted in a significant protective effect of FLCs as demonstrated by the lower degree of myocardial lesions and lower myocardial virus concentrations, and increased survival. FLC showed to inhibit viral replication directly, but lambda FLC supplementation also significantly induced IL-10 gene expression and tended to increase interferon-α and -γ in heart tissue [43]. In a murine model for whey induced cow's milk allergy, FLC concentrations significantly increased after tolerance induction prior to sensitization. This increased FLC production appeared to be essential for immune suppression after tolerance induction as it was demonstrated that antagonizing FLC by F991 after tolerance induction but before sensitization, attenuated the tolerizing effect of whey pre-treatment (Van Esch., PhD thesis 2011). In the same study, a single injection of FLCs of irrelevant specificity, one day before start of sensitization, mimicked the high dose tolerance induction. Finally, recent studies of suppressor T cells, distinct from regulatory T cells and induced by high antigen dose tolerogenesis, suggest that suppression may be brought about via secretion of exosomes. These exosomes suppress specific CS effector cells by delivering inhibitory miRNA and are possibly coated with FLC to provide antigen-specificity to target the exosomes to specific cell types (P.W. Askenase, *et al.* manuscript in preparation). Though making it more complicated, this immune suppressing ability of FLCs

should be kept in mind when interpreting and discussing the preclinical and clinical data described in this thesis. Besides a more thorough analysis of mechanisms underlying these immune suppressing actions, future studies should additionally explore a possible distinct role for FLC monomers and dimers in these responses. Speculating on this, one could hypothesize that FLC monomers are more prone to mediate suppressive functions because of their inferior binding capacity compared to FLC dimers. However, in this situation both isoforms have to be able to occupy FLC-receptors. Alternatively, distinct receptors or signalling pathways could be involved.

Concluding remarks

Immunoglobulin free light chains are physiologically present in different human body fluids and increases in FLC concentrations are found in multiple disorders. The functional relevance of these increases is still not completely understood. As described in this chapter, our research implicates an important role for FLCs in pro-inflammatory immune reactions in many different organ systems. These roles appear not to be restricted to the effects of FLCs on mast cells. Basophil activation can be enhanced by crosslinking of FLCs as well as described in this thesis, and immune reactions could be influenced by FLC-binding to neuronal cells and neutrophils.

Figure 1 schematically summarizes current knowledge on FLCs and its involvement in immune responses based on the studies summarized in this chapter. Since most evidence so far point to a crucial role for mast cells in FLC-mediated responses, the main focus of this figure is on mast cells. As described above, it was demonstrated that FLCs may synergize with IgE-mediated mast cell [77], and -basophil activation. Therefore, step *6C* from figure 1 might be of crucial importance in human disorders. As described in this thesis, several disorders show increased FLC concentrations whereas antigen-specific IgE (or other activating stimuli) cannot be detected. Speculating on this, this could mean that low amounts of antigen-specific IgE are involved in suboptimal mast cell activation, which is amplified by the synergistic effects of crosslinking antigen-specific FLCs. The exact mechanisms involved in these activation cascades are still unclear. Moreover, it has been shown that low-affinity antibodies can significantly enhance basophil degranulation upon co-stimulation with high or moderate affinity antibodies [89]. This finding could support a substantial influence of antigen-specific binding by FLC, which is in general of a somewhat lower affinity than IgE. In addition, IgE might act as a ‘capture antibody’, enabling lower affinity antibodies, including FLCs, to bind antigen more effectively.

Pivotal to a better understanding of FLC-mediated responses is the identification of a receptor for FLCs. The fact that a synergistic effect of FLC-crosslinking on basophil activation is only observed upon co-stimulation with anti-IgE, and not with C5a or fMLP (both

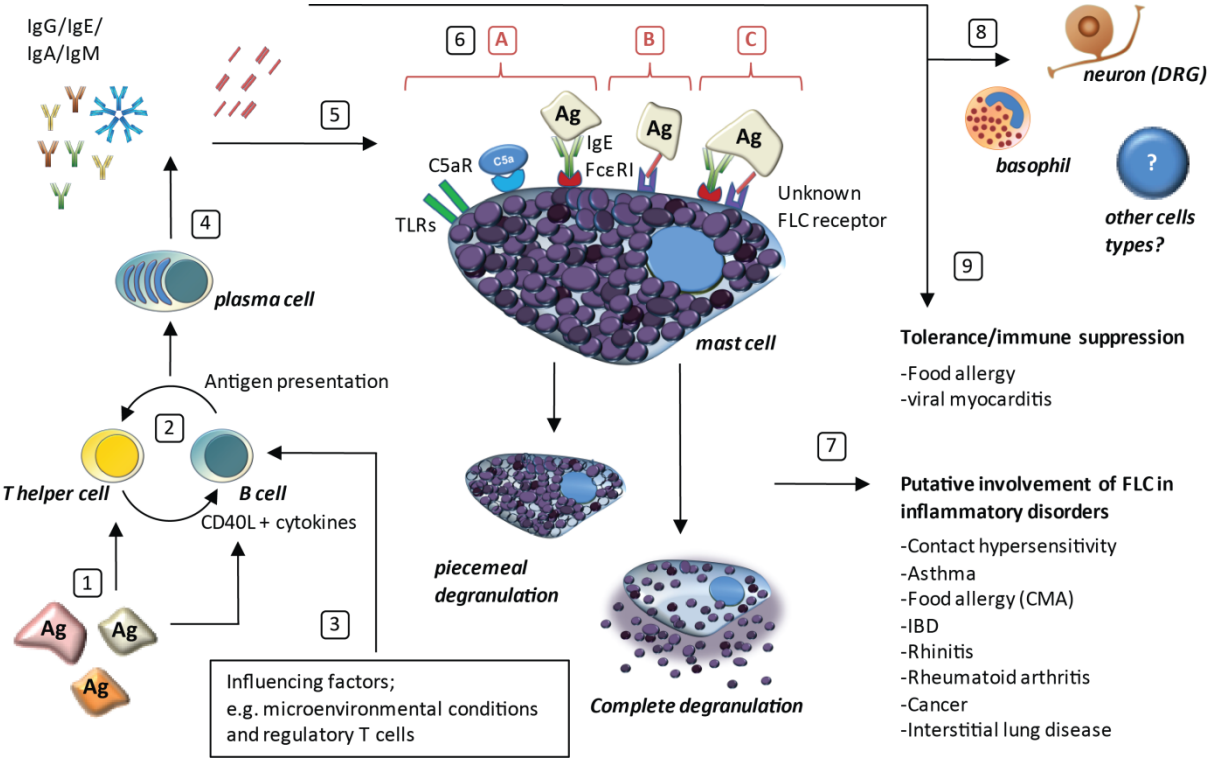

Figure 1. Schematic overview summarizing current knowledge on FLCs and its involvement in immune responses. 1. Exogenous antigens, or endogenous antigens in case of autoimmunity, either directly activate B cells (B-1 B cells, that are T cell independent in their activation and antibody production), or indirectly activate B cells via activated, antigen-specific T helper cells (**2**). **3**. Different factors such as environmental conditions and the presence of regulatory T cells (Tregs) can influence the reciprocal B cell-T cell activation (e.g. depletion of $CD25^{+}$ Tregs resulted in a shift from IgE to FLC producing B cells in a model for cow's milk allergy). Activated B cells differentiate into plasma cells which produce immunoglobulins and/or FLCs (**4**). The immunoglobulin isotype and the amount of FLCs produced depend on the activating stimuli and B cell subsets involved (**2** and **3**), and IgE, IgG and FLC can bind to specific receptors located on the mast cell membrane (**5**). Mast cells can be activated via multiple mechanisms; **6A**. Binding of IgE to their high affinity receptor (FcεRI) and subsequent crosslinking of receptor bound IgE is the best and most powerful known mechanism of mast cell activation. In addition, activation can be mediated via different Toll-like receptors (TLRs), and the complement component C5a receptor (C5aR). **6B**. Using different mouse models we demonstrated that FLCs are able to mediate antigen-specific mast cell activation. **6C**. In vitro mast cell activation assays support these in vivo data, but FLC-mediated activation can only be detected when mast cells are somewhat pre-activated using IgE crosslinking. Mast cell activation leads to either complete or piecemeal degranulation leading to subsequent initiation or progression of several inflammatory disorders (**7**). **8**. In addition, FLCs also bind to basophils and enhance its IgE-mediated activation, and affect dorsal root ganglion (DRG) function. Whether FLCs also bind to, and activate, other cell types is subject of ongoing research. **9**. In contrast to the immune activating effects of FLCs described above, several studies indicated the involvement of FLCs in suppressing immune responses in models for cow's milk allergy and viral myocarditis. Mechanisms underlying these suppressive effects are still unclear.

GPCRs), suggests that IgE- and FLC-receptors may share common intracellular events such as recruitment and activation of tyrosine kinases, which can cooperate to increase basophil activation.

Whether other activation stimuli can synergize with FLC-mediated mast cell and/or basophil activation is subject of current research. Evidence for the binding of FLC to mast cells is primarily based on results from preclinical models and on *in vitro* binding experiments on bone marrow-derived mast cells (BMMCs). So far, convincing evidence for FLC binding to mast cells in human tissue is only demonstrated in nasal mucosa tissue from rhinitis patients (**chapter 3**). FLC-binding to mast cells was not proven in CRS, IPF, HP, and RA patients. Although this could simply indicate that there is no FLC-binding to mast cells under most conditions, practical limitations might hamper the detection of FLCs co-localizing with mast cells. For instance, optimal immunohistochemical staining procedures for FLCs and mast cells do not correspond and both required mouse-anti-human primary antibodies. Furthermore, the diffuse pattern of FLC staining observed in many tissues could mask putative weak staining localizing on mast cells. Using flow cytometry for the detection of FLC-binding to basophils could largely avoid these disadvantages and resulted in the clear detection of FLC-positive basophils in human blood (**chapter 9**).

The functional involvement of FLCs in immune regulation is demonstrated in several preclinical models and the inhibiting effects observed after antagonizing FLC using F991 largely contributed to the current evidence. A disadvantage of F991 in its current form however is the short half-life which largely impedes the performance of long-term preventive or curative preclinical studies. In case of the melanoma model described in **chapter 8**, this problem could largely be circumvented by local administration. However, when studying the effectiveness of F991 treatment in a preclinical model for RA this is not realistic. Therefore, it is necessary to develop either newly generated FLC antagonists with a longer half-life or specialized delivery systems that result in a sustained F991 delivery after administration. A potentially interesting delivery system could be via biodegradable microspheres as described by A. Ghassemi (Ghassemi, PhD thesis 2011). The use of such microspheres could result in the prolonged presence of optimal concentrations of F991 which is probably necessary to attenuate chronic FLC-immune disorders.

In conclusion, current knowledge on the biological activity of FLCs obtained in *in vitro* research, and preclinical and clinical studies suggests that FLCs possibly affect multiple cell types, including mast cells in physiological and pathological conditions. Considering the pleiotropic biological effects FLCs, it would be interesting to investigate the therapeutic value

of FLC antagonists such as F991 in further detail. Nevertheless, the mechanism of FLC-mediated immunosuppressive actions needs further investigation as well, and should be considered when interfering with FLC antagonists.

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Appendix

Nederlandse samenvatting

Waarom dit onderzoek?

Ondanks verbeteringen in de gezondheidszorg is het aantal mensen dat lijdt aan aandoeningen waarbij ontsteking een belangrijke rol speelt de laatste decennia toegenomen in de westerse wereld. Aandoeningen die hierbij centraal staan zijn allergieën (zoals voedselallergie, astma, rinitis (bijvoorbeeld hooikoorts)) en auto-immuunziekten (zoals diabetes mellitus type 1, en multiple sclerose (MS)). De oorzaak van deze toename is niet geheel duidelijk maar kan mogelijk verklaard worden door de zogenaamde 'hygiëne theorie'. In deze theorie wordt een verband gelegd tussen een afname in het aantal infectieuze aandoeningen tijdens de eerste levensjaren, en de toename in het aantal allergieën en auto-immuunziekten. Daarnaast zijn veel mechanismen die een ontsteking veroorzaken en in stand houden slechts beperkt opgehelderd. Dit geldt niet alleen voor allergieën en auto-immuunziekten, maar ook voor andere aandoeningen waarbij ontsteking een belangrijke rol vervult, zoals bij veel vormen van kanker. Onderzoek naar deze mechanismen is daarom van groot belang om ontstekingsgerelateerde aandoeningen beter te kunnen begrijpen en behandelen.

B cellen en mestcellen

Het afweersysteem is een zeer complex systeem dat niet in detail in deze samenvatting besproken zal worden. B cellen en mestcellen zullen wel kort besproken worden omdat deze twee typen cellen een belangrijke rol vervullen in het onderzoek beschreven in dit proefschrift. B cellen zijn witte bloedcellen die in staat zijn om heel specifiek eiwitten (bijvoorbeeld allergenen) te herkennen en te binden. Deze binding kan vervolgens leiden tot activatie van de B cel, waarna deze verandert in een plasmacel. Een plasmacel is een celtype dat gespecialiseerd is in de productie van antistoffen, ook wel antilichamen of immunoglobulines (Ig) genoemd (figuur 1). Er kunnen verschillende typen antistoffen gemaakt worden, waaronder IgG en IgE. Deze verschillende typen antistoffen verschillen dusdanig van elkaar dat ze andere effecten in het lichaam kunnen veroorzaken. Zo is IgE het belangrijkste antilichaam dat betrokken is bij het opwekken van een allergie. Elke B cel produceert slechts 1 type antilichaam dat exact hetzelfde antigeen herkennen.

IgE is belangrijk bij allergie omdat het in staat is aan een mestcel te binden. Mestcellen zijn ontstekingscellen die zich door het gehele lichaam bevinden, maar voornamelijk in weefsels voorkomen die in direct contact staan met de buitenwereld, zoals de huid, longen en darmen. Mestcellen bevatten vele blaasjes die gevuld zijn met stoffen die een grote invloed

hebben op het ontstaan en het in stand houden van een ontsteking. De inhoud van de blaasjes komt vrij wanneer mestcellen worden geactiveerd (figuur 1). Bij astma leidt dit bijvoorbeeld direct in een vernauwing van de luchtwegen en dus benauwdheid. Hoe worden deze mestcellen geactiveerd? Hierbij is een belangrijke rol weggelegd voor IgE. Wanneer IgE moleculen op een mestcel aan een allergeen binden, dan vindt activatie van mestcellen plaats (figuur 1).

Echter, IgE is niet het enige eiwit dat in staat is om mestcellen te activeren. In veel patiënten die lijden aan een allergie of auto-immuunziekte is de aanwezigheid van geactiveerde mestcellen erg duidelijk, terwijl een rol voor IgE vrijwel zeker uitgesloten kan worden (hoofdstuk 2). Hoe de activatie van mestcellen in deze aandoeningen wel wordt veroorzaakt is vaak niet bekend. Inzicht in de rol van mestcellen in deze aandoeningen en de manier waarop activatie van deze cellen plaatsvindt is noodzakelijk om in de toekomst betere behandelingsmethoden te kunnen ontwikkelen.

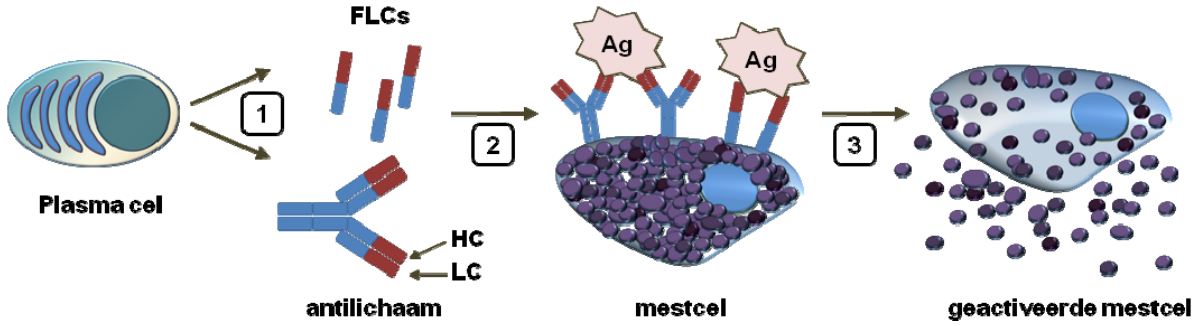
Immunoglobuline vrije lichte ketens

Een ander eiwit dat betrokken kan zijn bij de activatie van mestcellen is immunoglobuline vrije lichte keten. Lichte ketens vormen normaal gezien een onderdeel van een antilichaam. Antilichamen bestaan uit 2 zware ketens (figuur 1; HC) en 2 lichte ketens (figuur 1; LC). Beide type ketens hebben een specifieke regio dat kan binden aan bijvoorbeeld een allergeen. B cellen, die zoals hierboven beschreven verantwoordelijk zijn voor antistofproductie, kunnen ook alleen lichte ketens uitscheiden en deze worden zodoende vrije lichte ketens (free light chains; FLCs) genoemd.

Door gebruik te maken van muismodellen is op verschillende manieren aangetoond dat FLCs in staat zijn om een ontsteking te veroorzaken. Wanneer bijvoorbeeld antigeen-specifieke FLCs (ze herkennen stof X) in het bloed worden toegediend bij muizen en vervolgens het juiste molecuul (stof X) op het oor wordt aangebracht, dan ontstaat er een ontsteking die gemeten kan worden door middel van een toename in oordikte. Wanneer niet-specifieke FLCs worden toegediend ontstaat er geen ontsteking. Bovendien kan de ontsteking worden tegengegaan met een stof (F991) die heel gericht de werking van FLCs remt. De essentiële rol van mestcellen in dit ontstekingsproces is aangetoond door gebruik te maken van muizen die geen mestcellen hebben. Wanneer dezelfde proeven worden uitgevoerd met deze muizen dan kan er geen ontsteking worden opgewekt met behulp van FLCs.

De hierboven beschreven bevindingen hebben een nieuw ontstekingmechanisme in kaart gebracht dat mogelijk ook een rol speelt bij ontstekingsreacties in de mens. Hier is echter

nog niet veel over bekend. Het doel van dit proefschrift is om meer inzicht te krijgen in de productie, aanwezigheid, weefselverdeling en mogelijke functionele rol van FLCs in verschillende humane ziektebeelden zoals allergieën en auto-immuunziekten.



Figuur 1. Schematische weergave van FLC- en antilichaamproductie en het mogelijke effect op mestcellen. Indien een B cel voldoende wordt geactiveerd verandert deze in een plasmacel. Plasmacellen produceren vrije lichte ketens (FLCs) en antilichamen, bijvoorbeeld IgE (stap1). Antilichamen zijn opgebouwd uit 2 identieke zware ketens (HC) en 2 identieke lichte ketens (LC). Beide type ketens hebben een variabel deel (rood) dat betrokken is bij binding aan een antigeen. FLC en IgE zijn beiden in staat om aan mestcellen te binden (stap 2). Indien een antigeen wordt herkend en gebonden door IgE en/of FLC dat aan de mestcel zit gebonden, dan worden mestcellen geactiveerd. De moleculen die aanwezig zijn in de blaasjes van een mestcel worden dan uitgescheiden (stap 3). Deze moleculen zijn in staat een ontstekingsreactie in gang te zetten of in stand te houden.

FLCs in verschillende ziektebeelden

Luchtwegaandoeningen

Aandoeningen van de hoge en lage luchtwegen zijn bestudeerd door de aanwezigheid van FLCs te bepalen in vier verschillende ziektebeelden, namelijk: **a:** allergische rinitis (bijvoorbeeld hooikoorts) en niet-allergische rinitis met eosinofilie syndroom (afgekort; NARES) (hoofdstuk 3); **b:** chronische rhinosinusitis met en zonder neuspoliepen (hoofdstuk 4); **c:** overgevoeligheid pneumonitis (ontsteking van de lage luchtwegen) en **d:** idiopatische pulmonale longfibrose (waarbij bindweefselvorming in de long optreedt) (c en d zijn beiden beschreven in hoofdstuk 5). In alle genoemde aandoeningen zijn zeer duidelijk verhoogde FLC concentraties gevonden in het weefsel waar de ontsteking plaatsvindt. Ook in het bloed van de meeste patiënten waren FLC concentraties verhoogd ten opzichte van gezonde controles, echter deze verschillen waren minder duidelijk. Naast FLCs werden ook andere ontstekingsgerelateerde eiwitten gemeten, zoals IgE, IgG en tryptase. Tryptase is een van de eiwitten die voorkomen in de blaasjes van mestcellen en vrijkomt na mestcelactivatie. Bovendien werd naar de aanwezigheid van ontstekingscellen (bijvoorbeeld B cellen, plasmacellen, en mestcellen) gekeken in de ontstoken weefsels. In rinitis patiënten hebben we aangetoond dat mestcellen in het neusweefsel in staat zijn om FLCs te binden. Er was

geen duidelijke relatie te ontdekken tussen de mate van FLC productie en de specifieke productie van een bepaald type antilichaam (bijvoorbeeld IgE). Op deze manier hadden we meer te weten kunnen komen over bepaalde mechanismen die mogelijk betrokken zijn bij de productie van FLCs door plasmacellen.

Auto-immuunziekten: reumatoïde artritis

In hoofdstuk 6 van dit proefschrift wordt bewijs geleverd voor een mogelijke betrokkenheid van FLCs in reuma (reumatoïde artritis (RA)). FLC concentraties zijn zeer sterk verhoogd in de gewrichtsvloeistof en in het weefsel van de ontstoken gewrichten van RA patiënten. Bovendien hangen de gemeten FLC concentraties in de gewrichtsvloeistof samen met de gemeten concentraties in het bloed en nog interessanter, ook met de ernst van de ziekte.

Een relatief nieuwe behandelingsmethode voor RA is gericht op het terugdringen van het aantal B cellen in RA patiënten door middel van een zogenaamde anti-CD20 behandeling. In een deel van de behandelde patiënten is een duidelijke verbetering waar te nemen na behandeling. Door FLC concentraties in het bloed van RA patiënten te meten voor en na anti-CD20 behandeling, hebben wij aangetoond dat FLC concentraties alleen sterk dalen indien de patiënt ook een daling in ziekteactiviteit laat zien.

Kanker

Rondom veel tumoren ontstaat een ontstekingsreactie die een grote invloed heeft op de ontwikkeling van de tumor. Er is een nauwe samenwerking tussen tumor en ontstekingscellen: de tumor houdt de ontsteking in stand, en de ontsteking kan ervoor zorgen dat de tumor zich verder kan ontwikkelen. Mestcellen spelen in deze interactie waarschijnlijk een belangrijke rol en dit is uitgebreid beschreven in hoofdstuk 7 van dit proefschrift. In hoofdstuk 8 wordt de overvloedige aanwezigheid van FLCs beschreven in dat afkomstig is van verschillende organen van kankerpatiënten. Een uitgebreide analyse van borstkankerpatiënten laat zien dat aanwezigheid van FLC in het tumorweefsel gepaard gaat met een slecht ziekteverloop. Daarnaast hebben we in een diermodel aangetoond dat de tumorgroei zeer sterk afhankelijk is van de aanwezigheid van zowel mestcellen als FLCs.

FLC binding aan andere ontstekingscellen

Het onderzoek in onze onderzoeksgroep richt zich met name op het effect van FLCs op mestcellen. Echter, FLCs zijn ook in staat om aan andere cellen te binden. Toch is er weinig bekend over binding van FLCs aan andere celtypen en de mogelijke effecten hiervan. In hoofdstuk 9 wordt de binding van FLCs aan basofielen aangetoond. Basofielen zijn

ontstekingscellen die in het bloed voorkomen en zich kunnen verplaatsen naar weefsels waarin een ontstekingsreactie plaatsvindt. Basofielen worden steeds meer gezien als cellen die een belangrijke rol kunnen vervullen bij ontstekingsreacties zoals bij een allergie. In hoofdstuk 9 wordt ook aangetoond dat FLCs in staat zijn om de activatie van basofielen te beïnvloeden en dus ook via deze weg invloed op een ontsteking kunnen hebben.

FLC productie

Mechanismen betrokken bij de productie van FLCs door plasmacellen zijn slechts beperkt beschreven. Worden FLCs altijd uitgescheiden wanneer antilichamen worden geproduceerd, of wordt FLC productie heel nauw gereguleerd? In hoofdstuk 10 wordt een begin gemaakt met het achterhalen van mogelijke mechanismen.

Conclusie

De kennis die tot nu toe verzameld is over de effecten van FLCs is gebaseerd op *in vitro* onderzoek (experimenten uitgevoerd in een “reageerbuis” buiten het lichaam van een organisme), diermodellen, en op klinische studies met betrekking tot verscheidene aandoeningen in de mens. Al deze studies samen wijzen erop dat de productie van FLC sterk verhoogd is in veel aandoeningen waarbij ontsteking centraal staat en dat FLCs een effect kunnen hebben op meerdere celtypen (waaronder mestcellen en basofielen). Gezien de potentie van FLCs om ontstekingsreacties te veroorzaken is het zeer interessant om het resultaat te onderzoeken van toediening van stoffen die het effect van FLCs kunnen remmen (zoals F991). Zo wordt meer inzicht verkregen in de werkelijke bijdrage die FLCs leveren bij het opwekken of in stand houden van bepaalde ziektes. Bovendien kunnen op deze manier mogelijke nieuwe geneesmiddelen gevonden worden voor deze aandoeningen in de mens. In vervolgonderzoek moeten mechanismen die betrokken zijn bij de FLC-gemedieerde activatie van verschillende celtypen in meer detail bestudeerd worden.

Dankwoord

Bedankt, iedereen die de afgelopen 4 jaar direct of indirect heeft bijgedragen aan de totstandkoming van dit proefschrift! Kort maar krachtig en wel gemeend! Wat heb ik veel geleerd en een enorm leuke tijd gehad bij de Farmacologie.

Degene die ik hiervoor het meest wil en moet bedanken is Frank. Frank, eigenlijk vanaf mijn sollicitatiegesprek heb jij ervoor gezorgd dat ik me op mijn gemak heb gevoeld in de 'free light chain groep'. Ik heb ontzettend veel gehad aan jouw enorme hoeveelheid kennis, inzicht, gedrevenheid en optimisme. Je hebt altijd tijd voor een praatje of om te reageren op een mail. Dit is erg motiverend en naar mijn idee de perfecte manier om een groep te leiden. Bovendien draagt een 7-gangen diner van 'top chef' Frank of een heerlijke barbecue bij jou thuis hier uiteraard ook aan bij! Natuurlijk ben ik je ook veel dank verschuldigd voor alle moeite die je hebt gedaan voor de subsidieaanvragen voor een postdoc baan. Zonder jouw inspanningen en connecties was mijn vertrek naar New Haven in maart van dit jaar waarschijnlijk nooit tot stand gekomen.

Frans, mijn promotor, jou wil ik graag bedanken voor de mogelijkheid om op de afdeling Farmacologie te promoveren. Johan, graag wil ik ook jou bedanken ook al was je niet direct bij mijn onderzoek betrokken. Je bent altijd enthousiast over het 'light chain' onderzoek en hebt mij regelmatig betrokken bij interessante klinisch georiënteerde projecten van Danone. Dit vond ik erg leuk om te doen!

Iedereen enorm bedankt waarmee ik heb mogen samenwerken de afgelopen 4 jaar: Raymond Schiffelers, Ebel Pieters, Joel van Roon, Janneke Tekstra, Sarita Hartgring, Edward Knol, Els van Hoffen, Helma van Doorn, Rudi Hendriks, Lien Calus, Philippe Gevaert en Claus Bachert. Thank you for our successful and pleasant collaboration: Des Powe, Phil Askenase, and Moises Selman.

Dan iemand die zowel direct als indirect erg betrokken is geweest bij mijn zeer geslaagde tijd bij de Farmacologie: Bart! Niet voor niets mijn paranimf. Bedankt voor al je hulp in het lab, alle gezellige momenten in en buiten het lab, de 'oneliners' en de goede quotes (met name die over mannen, vrouwen, melk en wijn zal ik niet vergeten…). Caroline, mijn andere paranimf, je enthousiasme en gezelligheid maken het werk, de feestjes en het sporten altijd leuker! Bedankt hiervoor, ook al loopt het werk om deze reden nog wel eens wat vertraging op. Bart en Caroline, ook bedankt voor alle 'colavieux'; hopelijk volgen er nog een paar…

Bovendien waren de afgelopen jaren op de werkvloer voor mij een stuk gezelliger met de volgende personen: Lieke, Kim, Sas O, Sas B, Marco, Bastiaan, Daphne, Susan, Marije, Laura en natuurlijk ook Steffen. Bedankt voor de leuke tijd!

Eva, bedankt dat je me van Nijmegen naar Utrecht hebt gehaald! Ik heb er geen moment spijt van gehad. Hopelijk kunnen we elkaar over een jaar ook bedanken voor een geslaagd gezamenlijk avontuur in Amerika!

Curriculum Vitae

Tom Groot Kormelink was born on the 5th of March 1984, in Winterswijk, The Netherlands. He grew up in Groenlo where he also attended high school (Marianum comprehensive school) from which he graduated in 2002. In the same year, he started his study Biomedical Sciences at the Radboud University in Nijmegen. Tom finished his bachelor in 2005, and his master in 2007. Three months after graduating he started his PhD research project concerning the role of immunoglobulin free light chains in inflammatory diseases in the research group of Dr. Frank Redegeld at the University of Utrecht. At the end of 2011, this research resulted in the completion of this thesis. After finishing his thesis, Tom continued his research on FLCs for three more months as a part-time job at the group of Frank Redegeld. In parallel, he was involved in teaching of undergraduate students attending the College of Pharmaceutical Sciences in Utrecht. In March 2012, Tom will move to the US to work as a post-doc in the lab of Prof. Phil Askenase, at the Department of Internal Medicine, Yale University School of Medicine.

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