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DIPLOMARBEIT

CHARACTERIZATION OF TH2 CELLS AND

RELATIVE CHEMOKINE RECEPTOR EXPRESSION DURING DISTINCT PHASES OF

ALLERGIC ASTHMA

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ABSTRACT

IMMUNOLOGICAL MEMORY (IM) is the ability of the immune system to respond faster, more effectively and with greater magnitude to a previously encountered antigen. This ability is mediated by memory lymphocytes generated during first antigen encounter. Generation of memory lymphocytes is beneficial in recurrent infections because they provide long-lasting protection from pathogens. On the other hand, memory lymphocytes can also have a pathological role. The immune system of atopic persons has a tendency to react against harmless environmental antigens (allergens), like dust and pollen. Exposure of atopic individuals to allergens can lead to development of allergen-specific $CD4^+$ T helper 2 cells (Th2 cells) [2]. Allergen-specific Th2 cells elicit a pathological cascade resulting in elevated IgE antibody titers and an increased number of eosinophils, amongst other parameters [3].

Reactions to allergens are manifested in diverse ways, depending on the affected organ and potentially the allergen itself. A patient with allergic asthma (AA) suffers from recurrent episodes of wheezing, breathlessness and coughing due to a severe chronic inflammation of the airways, hyper-secretion of mucus, bronchial hyper-responsiveness and reversible airway obstruction [3]. The potential importance of memory Th2 cells in asthma is accentuated by the findings about T helper 1 (Th1) cells and cytotoxic T cells (CTLs) in mice that perpetuation of numerous chronic inflammatory disease states is possible through antigen-specific memory cell installation in peripheral tissue.

Mouse models of AA are well defined [4]. Sensitization and subsequent antigen aerosol challenge of mice induces an acute inflammatory response in lungs that resembles AA in humans [5]. After recovery from this acute episode of AA, recovered mice retain cell-infiltrates in lungs and display elevated titers of IgG1 and IgE antibodies in sera, when compared to naïve mice. Interestingly, infiltrates in the lungs of recovered mice contain antigen-specific memory Th2 cells. Earlier work has shown that these memory Th2 cells remain in the lungs for the lifetime of recovered mice and appear to be essential for disease relapse after antigen re-exposure [6].

OBJECTIVES This study aims to elucidate the role of chemokine receptors (CKRs) in the maintenance and migration of allergen-specific Th2 memory cells in the lungs of asthmatic mice. It will also focus on many genes that could serve to indicate the presence and activation of allergen-specific Th2 memory cells in the lungs, including Th2 cytokines and T cell activation markers. To examine the role of CKRs in maintenance of allergen-specific Th2 memory cells, gene expression was compared between naïve healthy controls and mice recovered from an acute asthma attack. To investigate the role of mentioned genes in the disease relapse, expression differences between recovered and mice that were re-challenged with allergen were compared.

METHODS We examined differences in CKR and selected marker gene expression between healthy mice (naïve), asthmatic mice recovered from acute disease (recovered), and normal asthmatic mice that are re-exposed to allergen (re-challenged) in a comparative gene expression evaluation of the whole pulmonary transcriptome via real-time PCR.

RESULTS TCR variable region α13 and the two monogamous receptors CCR6 and CCR9 were the only three genes that showed detectable difference in gene expression between naïve and recovered mice: while TCR α13 and CCR9 expression were increased, CCR6 expression was decreased in recovered mice compared to naïve mice. Expression levels of all Th2-specific cytokines, IL-4, IL-5 and IL-13, were immensely up-regulated after allergen re-challenge, while IFNγ showed a significant up-regulation between 4 and 6 h after allergen exposure of asthmatic mice. Several genes of secreted asthma promoting factors (TSLP, IL-33 and eotaxins-1 and -2) showed clear up-regulation, while specific genes of Th2 cells were either not affected (ST2) or down-regulated (CRTh2 and IL-25R). Evaluation of CKR expression after allergen re-challenge revealed different regulation patterns, while CCR1 and CXCR2 showed clear up-regulation from 2 h and CCR10 and CX_3CR1 showed clear down-regulation from 4 h after contact with allergen. We observed that a group of CKR genes did not react to the re-challenge at all (CCR4, CCR7, CCR9, CXCR1 and CXCR6).

CONCLUSIONS On the scale of the whole lung RNA vα13^{+} TCR sub-chain variant expression was a reliable marker for the presence of allergen-specific memory T cells in the lung tissue of asthmatic mice. Further, our results imply that CCR9 might be involved in the maintenance of these memory cells, which has to be further validated in subsequent experiments. Upon allergen re-challenge, pulmonary cytokine gene expression indicates elicitation of a strong Th2-mediated allergen-specific immune response within the first two hours after re-challenge. Gene expression pattern of diverse asthma and lymphocyte-related markers assisted in gaining insight in the early events after allergen re-challenge. Global pulmonary gene expression patterns of almost the whole panel of known CKRs was markedly changed after re-challenge. This change, however, was only permanent for CCR1, CxCR2, CCR5, CCR10 and Cx_3CR1. Additionally, we observed that there might be a limited number of distinct regulation pattern that CKRs obey as the lungs are re-challenged. One group of CKRs did not react to allergen exposure and gene expression alterations of these genes (CCR4, CCR7, CCR9, CxCR1 and CxCR6) might therefore not be necessary for the initiation of an asthmatic response. Yielded information should serve the design of specific pharmaceuticals and/or treatments for the cure from recurrent asthmatic phenotype upon contact with aeroallergens.

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ABBREVIATIONS

AA	**ALLERGIC ASTHMA**
IM	**IMMUNOLOGICAL MEMORY**
Ig	**IMMUNOGLOBULIN**
Treg	**REGULATORY T CELL**
FcR	**ANTIBODY HEAVY CHAIN RECEPTOR**
IL	**INTERLEUKIN**
OVA	**OVALBUMIN**
AHR	**AIRWAY HYPER-RESPONSIVENESS**
APC	**ANTIGEN-PRESENTING CELL**
TSLP	**THYMIC STROMAL LYMPHOPOEITIN**
TCR	**T CELL RECEPTOR**
Th2	**HELPER T CELL**
CTL	**CYTOLYTIC T CELL**
NKT	**NATURAL KILLER T CELL**
T-bet	**T-BOX EXPRESSED IN T CELLS**
ROR-γt	**RAR-RELATED ORPHAN RECEPTOR GAMMA T**
FoxP3	**FORKHEAD-BOX PROTEIN3**
STAT	**SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION**
CD62L	**L-SELECTIN**
CRTh2	**CHEMOATTRACTANT RECEPTOR-HOMOLOG ON TH2 CELLS**
IFN	**INTERFERON**
MHC	**MAJOR HISTOCOMPATABILITY COMPLEX**
CD62L	**L-SELECTIN**
mRNA	**MESSENGER RIBONUCLEIC ACID**

INTRODUCTION

IMMUNOLOGICAL MEMORY (IM)

An individual is called immune, when it is resistant to an outward or inward threat to a high degree. '*In munis*' from Latin – literally meaning 'freed from

obligations and taxation'• in ancient times – has adopted the modern tenor of 'being untouchable' and 'incapable of being harmed'. It is obvious that every organism is anxious to hold omnipresent noxious factors physically off it; if necessary in tolerable conditions to uphold physiological functions. In cases of immunodeficiency it fails, becomes fragile and acquires a noticeably short span of life in its natural habitats. In mammals, central players of immunity were recognized to be a heterogeneous group of cells, called immune cells, giving birth to the fields of immunology. It is one of current basic principles of immunology that immunity seems to possess a remarkable memory having structures of encountered proteins and other rather simple molecules imprinted that are not encoded in the genome of the organism. This recall-concept is closely intertwined with what is known to us as the adaptive recognition process of foreign, in comparison to self [7]. When 'awareness' about a foreign molecule sets in, this molecule is termed antigen in regards to the immune system. This immune system-antigen relation is the foundation of every immune response. After physical contact, intruded immunogenic proteins themselves are used for response elicitation to serve for their own elimination. As the antigen disappears, the reactive part of the immune system vanishes with it, and the original state of health is restored. In the body's interest only memory about the antigen's presence is remained without noticeable physical manifestation or aftermath. As a consequence of IM, the required type of memory immune response against a pathogen is induced faster, more adequate and with increased determination, upon repeated contact with its elicitor – the antigen. As a general rule, increase in efficiency to recognize proteins as belonging to pathogens and potential agents of disease increases immunity. Therefore, the vital concept of IM is to adapt to pathogenic environmental factors, to prevent disease. The adaptive capability of antigen recognition of the immune system is performed via production of cell membrane proteins called antigen-specific receptors rendering immune cells capable of reliably identifying antigens [8], and with assistance of other cell membrane proteins even in their pathologic context, i.e. viral, microbial, parasitic, and fungal.

•**Stowasser** Lateinisch-deutsches Schulwörterbuch**, Josef M. Stowasser,** M. Petschenig, F. Skutsch, Wien und München 1998 (Hölder-Pichler-Tempsky/Oldenbourg), XXXIV

Antigen-specific receptors can be found on the plasma membrane of T and B cells. These cells are offspring of hematopoietic cells from the bone marrow. When they mature, somatic base mutations and random segment recombination in antigen receptor gene sequences permit each lymphocyte clone to produce a transmembrane protein with a uniquely structured domain for recognition and antigen binding [9]. The recognition by lymphocytes is directed against one single antigenic determinant that is already established, as lymphocytes are still naïve to their antigen [10]. Recognition occurs because of the complementarity of the receptor's and antigen's surfaces that leads to their binding in a non-covalent nature [11]. The receptors are embedded in large complexes consisting of different peptides and various intra- and extracellular domains that act in concert to perform antigen recognition and signal transduction [12]. These events lead to the actual effectiveness of an adaptive immune response, when lymphocytes are activated, start to proliferate, and give rise to a multiple number of matured clones expressing one distinct antigen-specific receptor version. The population of activated antigen-specific lymphocytes then prompts the course of the initiated immune response into an adapted reaction. When memory cells are activated, the response is fast and intense, is part of immunological memory because it deals with immunogenic agents encountered before in the history of an organism, and immunizes the organism against the recognized antigen.

IMMUNOLOGICAL MEMORY RESPONSE – A DOUBLE-EDGED SWORD

Benefits from the acquisition of antigen receptor expressing lymphocytes during evolution were recognized early in human civilized history [13] long before peptides, cells or even pathogens were known. First survivors of small pox infections were observed to be unlikely to suffer from a recurrent episode of disease. Soon parents deliberately infected their children with blisters from marked small pox patients assisting them to become more resistant to the disease. After the first attempts to induce IM against small pox by non-threatening infections with cowpox [14], adaptive immunity was focused upon by numerous scientists – still ignorant of the existence of pathogens, to learn how to control this immunological mechanism by a procedure that is nowadays known as vaccination [15]. It was not known then that the acquisition of IM against a specific environmental factor was connected to

the emergence of novel persistent antigen-specific memory lymphocyte populations in the organism.

For every organism there are two important aspects for the development of IM: generation of an antigen-specific receptor repertoire built up by naïve lymphocytes and generation of antigen-specific memory cells forming an IM. Naïve lymphocytes are concentrated in the lymphatic and vascular system and are in steady inactive movement until they induce apoptosis. Before and after immune responses their number is roughly constant, what means that their homeostatic population as a whole proliferates as fast as it perishes [16]. Throughout an organism's life time, however, the size of the thymus (location of immature thymocytes and their maturation into functional T cells) fluctuates and mirrors generally the body's defense capacities [17]. While having its apex at puberty, the thymus atrophies with age, developing reciprocal in size and fat content. Memory cells, on the other side, are derived from activated naïve lymphocytes, can also be found in non-lymphoid tissue [18] – to become of major interest in this study – and tend to accumulate in variety along life time. It is this increasing variety, which brings a latent risk with it.

The specificity and normal activity of the antigen recognition event is crucial for an optimal state of health. Misdirected recognition will initiate immunological mechanisms that have harmful effects on an organism's physiological functions. There are several turning points in the life of lymphocyte progeny to pass control mechanisms, be activated and become memory cells [19]. Like everything in biology these control mechanisms are neither infallible nor universal. A failure in the selection process against self-directed antigen-receptors is the best known example, which can lead to the development of autoimmunity [20]. Consequently, proteins that are expressed somewhere at a site of the body receive the capability to induce an immune response, and as adaptive immunity is launched, IM is affected with severe long-term consequences for the inflicted organs. The connection between memory cells and *permanent cellular response* has drastic effects and ends often in the development of all kinds of pain states and dysfunction-preceding organ injuries. Development and perpetuation of *allergic states* and *installation of memory cells* in an organism reflects a more subtle connection of an IM-related pathogenicity.

ATOPY AND ALLERGY– PATHOLOGICAL ROLE OF IM

"I've had asthma before but I've never been through anything like this *(referring to a phase of exacerbation, author)* and it's been scary."
[Jo Jackson, leading British swimmer]

TENDENCY TO HYPER-REACT

Atopy is a Greek word derived from *atopos* meaning 'mislocated' or 'unusual'• and is used in modern medicine to describe immunologic hypersensitivity of individuals. The immune system has the intrinsic characteristic to react to any foreign substance. The reason for hypersensitivity phenomena is the hyperactivity of the immune system towards a more or less abundant matter from environment, markedly, when the pathologic context is missing. Exposure to diverse factors from the environment leads in patients suffering from atopy more frequently and more severely to development of immediate hyper-reactive immunological episodes. Today immunologists distinguish between at least four types of hypersensitivity [21]. The burden of atopic patients was strongly brought in connection with hereditary components [22]. In a yet unknown process this disposition – interestingly connected to elevated production of IgE [23] (discussed later) upon environmental antigen exposure [24] – can manifests itself in atopic allergy.

Depending on which organs are exposed to the responsible substance, the manifestation of atopic allergy leads to regular excessive responses of the eyes (allergic conjunctivitis), the nose (allergic rhinitis), the skin (allergic dermatitis), or the lung (allergic asthma). When the first experimental anaphylaxis phenomena were induced during immunization procedures, it was not clear what caused the increasing hypersensitivity in vaccine-treated subjects [20]. This transition to permanent hypersensitivity is based on the concept of IM and resembles very much the process of immunization.

•A patristic Greek lexicon, G. W. H. Lampe, Oxford University Press, 1961

ALLERGY MORBIDITY

[25] In history allergy has basically been a speculative description of the onset of periodic anaphylactic conditions induced by a vast spectrum of protein allergens. Charles Richet first brought the diverse symptoms of the disease into one term in 1901, when he noticed that the injection of proteins is able to definitely change the composition of the blood in an organism [26]. The name for it was derived from the Greek word 'allagei' meaning 'change [from original state]' and implicates Richet's observation that allergies are established by the two-step process of sudden allergen-linked sensitization and following sequences of anaphylaxis induced by the allergen's consumption. Later, the link to these immunologic events was drawn to the presence of elevated concentrations of allergen-specific immunoglobulin (Ig).

A central pillar in allergology since the late 1980s is that allergy is based on 'chronically activated helper T cells driven by antigens to a perpetuated inflammatory response in and around bronchi through the release of T cell-derived lymphokines' [27]. Until now the patients' switch from healthy to allergy could not be successfully restored in a general approach. The swiftness in transition from healthy to allergic, as well as, from normal to anaphylactic was the most impressive problem for the institutions dealing with the recently epidemic disease. The fast spreading of allergies in the western population in the midst of the 20th century, especially amongst children, seemed for many scientists to have gone hand-in-hand with the fast developments in modern medicine. Many theories evolved trying to connect the conditions of the new environment of modern societies with allergy that, in fact, has always seemed to be majorly directed by environmental factors [28]. Richet himself confidently drew the link of the emergence of the broad use of needled syringes by modern physicians with the large scale sensitization of the population to allergens, accusing the accidental entering of

Figure 1. In 1906, Clemens von Pirquet coined the term '*allergy*' for the new antibody-mediated hyper-sensitivity disorder, described by Richet. Memorial to the Austrian physician in the General Hospital of Vianna.

allergenic proteins through direct ways into the bloodstream (like it has never happened in human history before) to be the major cause for the wide spreading. Nowadays, while some experts trace back establishment of complex allergic states to a developing sensitivity driven by specific altered allergenic food components released during digestion [29], other scientists and MDs have put the blame on the extreme hygiene practiced by western populations for bringing the adaptive immune system, especially the defensive system against helmintic [30] and ectoparasitic [31] intrusions, out of balance. The hygiene hypothesis implicates lacking or incomplete development of regulatory mechanisms based on T regulatory cells (Tregs) to result into a bias towards perpetuated inflammatory responses to non-pathogenic environmental factors [32]. These and other theories about the development of allergies, however, could not build up a complete hypothesis about the genesis of the chronic inflammations, obviously, due to inoculation with innocuous proteins.

The most relevant concern of medical institutions concerned about allergy is, why some people develop allergies, while others are not inflicted by the handicaps of these conditions. One intuitive assumption leads to the idea of people's immune systems' divergent abilities in antigen-presentation [33]. Additionally, the different molecular breakdown of an ingested proteinaceous allergenic factor could result directly in changes in its immunogenicity. The focus of this study, however, will not be about how the differences between people can be found in the history of individuals and how they encounter environmental factors, or about what factors drive the immune system into launching a permanent immune response against abundant but harmless antigens. Our agenda will be foremost about how allergy-triggered inflammation is perpetuated and promoted, in spite of regulatory mechanisms.

IM AND ALLERGY

Immune responses are not elicited in an all-or-nothing nature, but biased towards alternative response types through the effects of signaling factors, i.e. cytokines, and antigen-presentation. In other words, influencing the development of cells that are responsible for response type elicitation creates a response bias. Allergic response elicitations are displaced (due to the harmlessness of allergens) but complete and include generation of antigen specific memory cells. *Whether an allergic response is generated or not is complex* and depends primarily on amount, type, uptake-frequency and -route of the allergen. Furthermore, concomitance of additive effects of Toll-like receptor ligands and other sensitization-enhancing agents contribute to allergic response initiation [34]. As counter activity, proper functionality of regulatory mechanisms, including Treg activity and TGFβ or IL-10 production, dictate the development of tolerance and immune response remission [35].

A crucial rationale about the involvement of IM in allergy perpetuation is a profound event in the development of allergies, which is the induction of sensitizing allergen-specific Ig production, generally referred to as *sensitization*. The binding specificity of the antibody is maintained, while the physiological aspects mediated by the Ig heavy chain are completely altered upon antigen binding. This altered Ig production happens due to the endocrine Ig heavy chain recombination-promoting effects of IL-4 and IL-13 on B lymphocytes produced by Th2 lymphocytes, mast cells [35] and eosinophils [36]. Therefore, an allergen must have the ability to elicit a Th2 cell response, to enable the Ig heavy chain isotype class switch to a sensitizing isotype and *memory* B cell formation to occur. In sensitized organisms there is consequently a permanent production of antigen-specific Ig with a sensitizing heavy chain isotype e.g. epsilon type – notably, a mural homolog to human IgE is IgG1. Newly formed memory B cells migrate throughout the whole lymphatic system residing partially in random lymph nodes, until they eventually rest in the bone marrow, where they continue the secretion of antigen-specific Ig that keeps the allergic organism sensitive to specific allergens for life [37]. For the investigation of the different phases of allergic asthma this study used purified chicken egg ovalbumin (OVA) to induce sensitization to OVA.

IM AND ALLERGIC DISEASES

[38] Allergen exposure in a sensitized organism leads to the activation of mast cells and basophils majorly through cross-binding of epsilon heavy chain antibody receptor 1 (FcεR1) on their cell surface. In sensitized organisms this activation through FcεR1 cross-binding is possible because of the presence of allergen-specific IgE antibodies binding FcεR1. Allergen can bind to these antibodies, and terminally, bring FcεR1 molecules to each other's proximity through more IgE-antibody interaction.

Receptor cross-binding of FcRs on the surface of mast cells and basophils leads to activation and the release of granulocyte mediators, like histamine [39] and other inflammatory cytokines. Besides chemical agents, also mechanical forces, oxygen or nutrient deprivation, and temperature extremes can trigger mast cell degranulation, leading to acute local inflammation [40]. The immediate reactions lead amongst others to infiltration of neutrophils into the site of allergen exposure within minutes, after causing typical allergic tissue responses, e.g. vasodilation, smooth muscle constriction, increased mucus secretion and increased vascular permeability [41]. As these complementing cascades are elapsing, macrophages and epithelial cells release factors mediating local recruitment of leukocytes that indicates the upcoming interference of another part of the immune system, basically composed of responding lymphocytes. A pointer is the evident $CD4^+$ T helper cell activation in the peripheral blood of allergic patients during exacerbations [42]. These cellular allergy-specific processes are visible at a histological level, due to profound influences on the chemical and functional qualities in allergen-exposed tissues of sensitized organisms. One of the consequences of altered microenvironment is the recruitment and activation of inflammatory cells, i.e. eosinophils.

The regulatory process that determines how the immune system is going to react in coordination to any substance in the organism is the integrative event of antigen-presentation. This remarkable process is mediated during direct physical interaction via the complex immunological synapse, when signals are sent back and forth between diverse types of leukocytes [43]. Antigen-presenting cells (APCs) prime and promote proliferation of antigen-respondent leukocytes, or induce tolerance [44, 45]. Diverse regulatory functions of DCs and the swift activation of allergen-specific memory leukocytes are a crucial focus for this study because they promote a type 2 T cell response elicitation and prolonged phases of allergic asthma. DC implication is further accented in the role of thymic stromal lymphopoeitin (TSLP). TSLP is a ligand for the IL-7R-like TSLP receptor and is expressed by epithelial cells, DCs and T helper cells in peripheral lymphoid tissues [46] synergistically regulated by TNF-α and IL-4 [47]. One of TSLP functionalities is to drive Th2 responses via involvement of DCs [48], and also to activate myeloid DCs towards driving middle-affinity $CD4^+CD25^+FoxP3^+$Tregs to high-affnity [49]. TSLP might promote progression of the course of asthmatic reactions because it induces Th2 cytokine production in vitro and activates eosinophils that express TSLPR constitutively [50].

After their activation, lymphocytes experience a vast cytokine-induced proliferative phase that amplifies their effectiveness. IL-2 was the first identified cytokine found in 1976 [51]. It was shown to be predominantly expressed by T cells as a major T cell growth factor and promotes proliferation and activation of IL-2 receptor expressing cells [52]. The IL-2 receptor is composed of three subunit chains named IL-2Rα (or CD25), IL-2Rβ (or CD122) and IL-2Rγ (or CD132) [53]. The β- and γ-chain are constitutively expressed on the surface of T cells and form a dimer with a low affinity to IL-2. During immune responses IL-2α, however, is one of early up-regulated, extensively glycosylated membrane proteins that has the ability to capture IL-2, presumably induce conformational changes in its structure and bring it to proximity of the preformed IL2β/γ dimer that has now a 100-fold higher affinity to its ligand [53]. This increase in IL-2 receptor binding efficiency supports CD25 expressing lymphocytes with a strong proliferation impulse.

Sensitization requires activation of allergen-specific lymphocytes after exposure to allergen. This process involves the primary up-take of allergen, followed

by fast allergen internalization within minutes and processing by APCs [54]. Internalized antigens are transported to proteolysing compartments of the endoplasmatic reticulum, where they are processed to peptide fragments attached to immunodominant sequences that bind to major histocompatibility complex (MHC) II molecules [55]. These newly formed complexes are transported to the cell's outer-membrane and cause with other molecules the stimulation of antigen-specific receptors of naïve and memory T cells, called T cell receptors (TCRs) [56]. The priming event of T cells during sensitization can be reduced to MHC-TCR interactions in membrane complexes supported by the involvement of different membrane-bound cofactors [56]. This leads to signal transductions, and notably, activation and differentiation of mature naïve T helper cells inside lymph nodes, sometimes referred to as Th0 cells [57]. Memory cell activation still seems to be more obscure to these days.

There are two major groups of mature T cells; helper and cytolytic T cells. Each T cell lineage is restricted to be activated by one class of MHC. In other terms, each lineage is presented with antigen sampled from another source. These are MHC class I^+ cells presenting cytosolic peptides to $CD8^+$ T cells; or professional APCs sampling endo-/pinocytosed peptides presented in MHC class II context to $CD4^+$ T helper cells. Therefore, when these two aspects are considered, it is not surprising that allergens induce humoral helper T cell responses. In the case of *allergy*, the idea of response elicitation by a helper T cell population to serve to eliminate protein allergens is sound, while the pathologic process taking place upon the presentation of antigens that otherwise have no pathological effects on the organism, is rather not.

In exposed tissues of sensitized individuals allergen induces activation of Th2 memory cells via antigen-presentation and suppresses cellular defense mechanisms. Importantly, instead of neutrophilia, Th2 effector cell proliferation and type 2-specific cytokine release promotes diverse *tissue responses* ***and*** *inflammation*, while enhancing eosinophilia [58]. The implication of Th2 cells in the promotion of allergies is far-reaching and extensively studied.

The transition to hypersensitivity is linked to an established adaptive immune response and has great implications with the concept of IM. The significance and adaptability of IM is accented in the concept of the atopic march [59]. The reason why this IM mechanism remained unmentioned here so far is that IM subtly installs in diverse sites of the sensitized organism with every exposure to allergen, while it does not need to show signs of allergies at all. The stepwise installation of type 2 immunity against repetitive allergen exposure in peripheral tissues reached more or less the level of a scientific fact. A synergistic implication of Th17-type responses is under discussion and might deliver further explanations for distinct phenomena during asthmatic reactions [60]. It is assumed that activation of IM differs from the naïve pathway. Memory responses might depend on less cofactor stimulation through greater TCR responsiveness [61] and might have the ability to be elicited outside lymph nodes. When hypersensitivity afflicts the lungs, bronchial asthma appears as an inflammatory disease of the airways, accompanied by the release of diverse chemotactic factors and cytokines, amongst others [62]. Signaling of the latter two leads to increased numbers of effector cells in asthmatic lungs that are typical for Th2-type responses [63].

CHARACTERISTICS OF ALLERGIC ASTHMA: **INCREASED AIRWAY REACTIVITY, DECREASED PULMONARY FUNCTION**

A strong hint that asthma can be triggered by allergic responses has been the fact that a large number of asthmatics are coincidently atopic. Furthermore, the occurrence of asthma amongst atopic schoolchildren is significantly higher than in non-atopics of the same age [64]. The fact that the number of the asthmatic-and-atopic coincidence is around 50%, however, indicates that atopy cannot be the only reason for the development of asthma [65], even though, biopsies from non-atopic· and occupational asthmatics showed clear immunopathologic similarities in allergic asthmatic lung biopsies at the histological level in studies from the late 1990s [66]. Even though the causes might be different, the processes that take place are, therefore, very similar in all forms of asthma. Cell infiltration, airway obstruction and tissue remodeling are the three major consequences of chronic

· intrinsic – supposedly self antigen-driven

asthma in humans and cause all diverse symptoms of AA throughout its different stages of severity [67].

Two prominent asthma characteristics are *airway remodeling* and *AHR*. Following inflammatory processes in the lungs, they are result of damaged structures of tissues, amongst others, by cytotoxic granules and lipid mediators released by eosinophils [68]. During the extended remodeling process transient changes in the lung tissue occur with alternating phases of acute disease and recovery that become permanent with increasing severity of disease [69]. Remodeling includes that epithelium structure changes, extracellular matrix proteins and collagen are deposited into sub-epithelial basement membranes, vascularity increases, and smooth muscle hypertrophy and hyperplasia occur [70]. These changes might compete with the tissues' mechanical flexibility and lead to narrowing of the airway ducts.

Simultaneous to the process of tissue damage, nerve endings are affected leading to bronchoconstriction [71], and furthermore to AHR [72]. The severity of asthma can routinely be monitored via bronchial challenge tests followed by spirometry, to quantify pulmonary function. For the challenge either histamine or methacholine is orally inhaled leading upon binding to their receptors, H1 and M3 respectively, to narrowing of the airways. The extent of narrowing is dependent on the degree of bronchial responsiveness. Then volume (lung capacity) and flow of air that the patient can inhale and exhale (airway resistance) is mechanically measured by plethysmography and interpreted. The reciprocal value of lung resistance to air flow is called respiratory conductance and gives profound information about pulmonary function. Frequency and steepness of breathing can be examined in oscillatory testing. Asthmatics are hyper-responsive to pulmonary stress and react therefore with shortening of breathing frequency upon the administration of methacholine. Since their airways narrow during acute attacks, they are not able to take deep breaths without wheezing or coughing.

MEMORY TH2 CELLS PERPETUATE ALLERGY AND ASTHMA

Strong evidence is accumulating to favor the implication of allergen-reactive type 2 T helper lymphocytes for playing a pivotal role in the installation (sensitization) and perpetuation of allergic inflammatory cascades [73]. While it is not known which different mechanisms induce emergence of different types of helper cells [74]; allergic immune response is held to be driven by generation of effector Th2 cells [75] showing altered expression of surface receptors and ligands, as well, as production of cytokines [76].

Whichever cause of allergies there might be, IM is not only responsible for the installation of asthma. Taking considerations a step further it creates another outstanding characteristic of allergic diseases: allergy perpetuation [77]. Regardless of the type of response, every immune response is primed by ways of innate immunity. Therefore, the involvement of deregulated antigen presentation is not an unthinkable cause for allergy perpetuation. To test this, an experiment to inhibit antigen-presentation over an extended time, after remission of an allergic response could show the effect of antigen-presentation on IM perpetuation. When indications of lowered relapse potential after local allergen exposure, due to this treatment, occur, the permanent presentation of allergen in the body could be the basis of IM maintenance. However, the most popular mechanism of upholding the ability to respond to a specific antigen is considered to be an intrinsic homeostatic characteristic of IM on a cellular basis [78].

In this study, establishment of allergic asthma in sensitized mice is achieved through local allergen exposure to the lungs with OVA aerosol. Past studies could show that OVA-specific memory cells can be detected in the lungs of asthmatic mice for a lifetime [6]. The same study showed that extraction of $CD4^+$ populations from lungs of mice that recovered from an acute asthmatic disease induced the production of Th2 cytokines when stimulated with OVA in vitro and that allergen re-challenge in vivo caused all characteristic signs of asthma relapse, even years after they were sensitized. Splenic $CD4^+$ cells from sensitized rats also induced passive asthmatic disease, after being transferred into healthy naïve rats [79]. OVA-specific $CD4^+$ cells from lungs passively transferred allergic disease to naïve recipients upon

OVA aerosolisation (private communication, M. Epstein). When an adaptive immune response against a specific antigen is elicited for the first time, competitive cascades are initiated that lead to distinct types of reactions. What type of adapted response is required to be elicited depends on the large scale on the localization and species of the pathogen and which T helper cell lineage is induced to develop. In contrast to early reactions in sensitized organisms that possess systemic allergen-specific memory plasma cells that cause elevated presence of allergen-specific IgE antibodies, allergen-specific Th2 cells orchestrate late events of allergic reactions.

To understand how asthma develops, the event taking place minutes *after* the immediately apparent and antibody-induced hypersensitivity paradoxically plays the crucial role in the establishment of asthma. The central paradigm of asthma is that severity of allergic reactions increases detrimentally with every further immunization step. Thus, Richet's termination is justified who observed that during allergy development the immune system is moving 'away from protection' (ana-: away from; phylaxis: protection). While the term aphylaxis would have implicated a lack in immunity, the subtle deflection in the term anaphylaxis leaves enough space for another interpretation – that increasing sensitivity is the cause for allergic disease.

When allergen enters the body of allergic individuals immediate and late allergic phase reactions can manifest in diverse ways and severity. Allergies are unpleasant and reduce conceivably an allergic person's quality of life, indifferently, where allergies break out in the organism. The consequences of the states that allergies induce have to be understood by patients themselves, as well as, by outsiders. The severity of allergy can collectively be sympathized in the case of asthma, when the responses concern the lung.

ALLERGEN-SPECIFIC HELPER 2 TCELLS AND DISEASE PERPETUATION

All aforementioned pathophysiologic aspects of chronic allergic disorders result in pathological effects that are connected to either cytokines and chemokines produced by Th2 cells or to cytokines and chemokines produced in response to the presence of Th2 cytokines or terminally as a reaction to Th2-related effects on the tissue [81]. Until the beginning of the 21st century the strongest hypothesis about the

molecular mechanisms included the basic events of IL-4 mediated IgE synthesis, maturation and sensitization of mast cells and basophils and their activation during acute allergic attacks, and IL-5-mediated eosinophil infiltration [35]. IL-13 acts synergistically to IL-4 as an Ig isotype class switch promoter, and seems to mediate additionally tissue related alterations to eliminate extracellular protein antigen from the affected tissue. Amongst other markers, Th2 major transcription factor GATA3 over-expression, in opposition to Th1 counterpart Tbet down-regulation, can be observed in mural asthmatic airways.

In this case allergen exposure in the lungs of sensitized individuals induces the accumulation of Th2 memory cells. In chronic asthma elevated levels of allergen-specific Ig antibody production by effector/memory B cells and the formation of allergen-specific effector/memory Th2 cells residing preferably in lymph nodes and the chronically exposed tissues are permanently established. The common way to distinguish differences between naïve and memory cells is by determining different phenotypes of their plasma membranes [82]. Memory cells express low levels of L-selectin (CD62L) and CD45R isoform CD45RB and high levels of CD44 ($CD44^{hi}$ – $CD45RB^{lo}$ – $CD62L^{lo}$), when compared with naïve cells ($CD44^{lo}$ – $CD45RB^{hi}$ – $CD62L^{hi}$). Effector cell are characterized as $CD44^{hi}$ – $CD45RB^{lo/hi}$ – $CD62L^{lo}$. However, there are other memory-specific surface molecules that are supposed to be Th2 cell and Th2 response-specific (see below).

TCR. Secondary antigen recognition by T cells is mediated via heterodimeric cell-specific antigen receptors consisting of an α- and a β-chain. Each chain possesses two Ig-like domains, one variable and one constant, what makes them members of the Ig-superfamily. The part of every TCR chain gene that has a set of variable regions is comprised of an isotype domain repertoire – in between non-coding parts of the genome – that is shuffled together during random recombination processes. Even though, interaction with antigen was identified to be performed by three determined domains of the variable region – that additionally experience somatic mutation during lymphocyte differentiation – the optimal strength of interaction is of a long but weak, un-covalent nature. Activation of T cells through antigen presentation requires the assembly of the TCR complex – most importantly CD3 and ζ - and stable, long-term physical and signaling interaction with APCs. CD3

plays a direct role in MHC binding and is essential for antigen-driven activation. Signal transduction, however, is majorly performed by disulfide-linked ζ molecules via a long cytoplasmic region.

CD4. This glycosylated surface molecule with four immunoglobulin domains can be found on different types of immune cells and is widely used for the discrimination between $CD4^+$ T helper and $CD8^+$cytolytic T cells (CTLs) [83]. It is not expressed on $CD8^+$ natural killer T cells (NKTs). In T helper cells CD4 is a co-receptor that assists TCRs during antigen presentation to activate and determine T helper cell maturation. CD4 has been shown to interact with SPG21 (involved in T cell activation repression), Lck (a tyrosinphosphatase that starts a phosphorylation cascade leading to an activating signaling cascade) and UNC119 (enhancing T cell activation) [84]. In this study, CD4 expression will be used to monitor the presence and migration of Th2 cells in the lungs of asthmatic mice after allergen exposure.

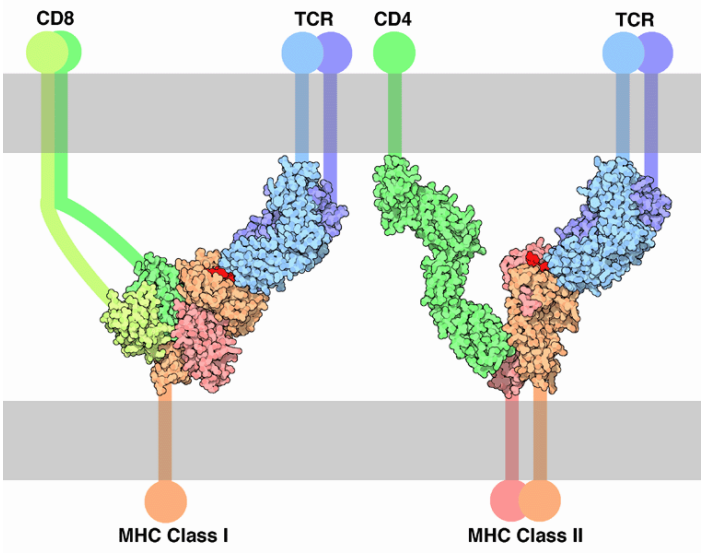


Figure 3. Position of CD8 and CD4 (depicted green) in the interaction of TCR (depicted blue) with the antigen-binding (antigen is depicted red) Major Histocompatability Complexes (depicted orange). CD4 is expressed on T cells to enable MHC class II interaction, while CD8 forms a complex with TCR to interact with MHC class I; therefore, the term MCH class II restriction for T helper cells. Illustration by David S. Goodsell of The Scripps Research Institute•.

•http://www.pdb.org/pdb/static.do?p=education_discussion/molecule_of_the_month/pdb63_1.h

GATA3, STAT6. These two molecules are main targets for the generation of Th2 response deficient knockout mice because of their crucial role in the stimulation of Th2 cells. Signal Transducer and Activator of Transcription 6 is member of the STAT family involved in the regulation of differentiation and survival of cells. Studies could show that STAT6 is able to induce high levels of GATA3 [85]. GATA3 was shown to be a Th2 specific member of the GATA sequence-binding transcription factor family [86]. Interestingly, it was shown to induce IL-4, IL-5 and IL-13 secretion, besides T cell development [87].

The specification of T helper cells is controlled by regulatory activity of type specific transcription factors [88]. So, an interesting aspect of GATA3 is its relation to T-box expressed in T cells (T-bet), RAR-related orphan receptor gamma t (ROR-γt), and forkhead-box protein3 (FoxP3). GATA3 is generally considered as the major regulator for the differentiation of Th2 cells, as T-bet is for Th1 cells, ROR-γt is for Th17 cells and FoxP3 is for Tregs. This means when naïve T cells are exposed to IFN-γ during antigen-presentation, T-bet expression is induced by STAT1, which drives Th1 cell differentiation and IFN-γ production to create a positive amplification loop for Th1 development. FoxP3, however, induces the development of antigen-specific Tregs in the thymus, as well as, the acquisition of their suppressive functionality [89].

IL-4, IL-5, IL-13. Th2-cytokine effectiveness is consistent with the observed Th2 predominance in allergies as result of numerous studies. IL-13 is active via actions on epithelial and smooth muscle cells. Direct interaction of T cells and smooth muscle showed increased ability of contractility in the presence of acetylcholine after interaction. Knock-out and blockage of this gene result in a significant decrease of asthma phenotype [90]. Together with IL-4 it activates B cells and initiates IgE-class switch [91]. Soluble receptors that neutralizeIL-4 and IL-13 could reverse AHR in mouse models. Each cytokine affects serum IgE and airway eosinophilia differently [92]. Mast cells, basophils and eosinophils themselves were also shown as potential sources of Th2-type cytokines [93, 94] and are associated with IL-4 and IL-5 involvement in the recruitment of Th2 cells. IL-5 acts primarily on bone marrow cells to support formation of eosinophils from G-CSF-pulsed blast cells and their exodus into the body [95]. IL-5 was also shown to directly promote eosinophil recruitment and activation and prolonged eosinophil survival [96].

ADDITIONAL MOLECULES THAT CHARACTERIZE MEMORY TH2 CELLS

ST2. This receptor is a member of the IL-1R superfamily and has at least two types of gene products, a soluble isoform and a transmembrane-isoform called ST2L. Previous studies showed that in asthmatic humans ST2 is up-regulated in serum and might influence the course of asthma development [97]. The soluble form might serve as modulator of Th2-mediated inflammations reducing acute exacerbation. **IL-33.** Intranasal administration of IL-33 induces eosinophilia and increasing levels of IL-5, IL-33, IgE, mucus secretion and hyper-responsiveness in the lungs of mice [98]. This is a strong indication for its participation in development of asthma from an early stage. Studies show that IL-33 is capable to directly induce eosinophil differentiation from hematopoietic progenitor cells and activate them promoting eosinophil airway inflammation [99] via the membrane-bound receptor ST2. While stromal cells constitutively express IL-33, mast cells only release IL-33 upon IgE-dependent activation [100] indicating that there is an allergy-prone function of IL-33.

IL-25 and IL-25R [101]**.** IL-17Breceptor (also known as IL25R) is able to bind two ligands of the IL17-family, IL-17B and IL-25 (also named IL-17E). IL-25 is expressed by activated Th2 cells, eosinophils and mast cells. It induces Th2 cytokine production and actively inhibits IFN-γ and IL-17 and therefore has an extensive ability to promote a Th2-biased response. IL-25/IL-25R signaling is, therefore, directly involved in the pathogenesis of allergic inflammation and was shown to induce IL-4, IL-5 and IL-13 up-regulation, eosinophil infiltration, mucus production in the lung, and AHR upon nasal administration. Interestingly, this cytokine was not only shown to be involved in type 2 immunity induction but also to be a mediator in its regulation by influencing levels of IL-17 and IFN-γ production [102].

CRTh2. Chemoattractantreceptor homologous expressed on Th2 cells is a G-protein coupled receptor binding Prostaglandin D2• with high affinity. It signals chemoattraction of Th2 cells and eosinophils and promotes Th2 cytokine secretion even in the absence of allergen. Prostaglandin D2 is an arachidonic acid metabolite and is released by mast cells during allergic responses [103].

• an early mediator of asthma development

THE CHEMOKINE SYSTEM IN AA

We have discussed so far that a genetic predisposition for developing any form of hypersensitivity is referred to as atopy in medical terms, while allergies have the tendency to develop on diverse ways into hypersensitivity of distinct parts of the body. Allergies have characteristic manifestations, which have strong correlation with the effectiveness of Th2 cells that seem to maintain allergen-specific memory cell populations and contribute to allergy perpetuation. IM is a sophisticated strategy to retrieve information about pathogens. This would physiologically only make sense in sites where it is required. Current knowledge about the cellular basis of IM acknowledges certain homeostatic populations of lymphocytes **strategically positioned** to fight life-threatening factors with maximal efficiency in the exposed tissue. Therefore, we can assume that localization of IM-building lymphocyte populations is crucial for advanced defense capacities of an organism, reflected by accumulating allergen-specific Th2 memory cell populations in lungs of asthmatic individuals.

Chemotactic cytokines, or chemokines for short, are small peptides involved in leukocyte chemotaxis, as well as alterations in ion fluxes, integrin avidity, cell shape, secretion of lysosomal enzymes, and production of superoxide anions. Chemokines are mostly soluble but there is evidence that some types (if not most types in vivo)can be immobilized by proteoglycans[104] helping to build up gradients on the endothelial glycocalix and extracellular matrices preventing them from being washed out by circulation. Since their first description in 1987• they were recognized as important factors for the pathogenesis of many human diseases and markers of disease onset, progression and remission. They belong to the chemokine superfamily, a group of 'proteins with chemoattractant properties and cytokine-like activities' [105]. They are divided into four groups according to the positioning of the first two highly conserved cysteins (C-C, C-X-C, X-C, and C-X_3-C). Much information about specificities and significance of chemokines could be acquired in the last

• Yoshimura, et al. reported interleukin 8 purification as discovery of the first identified chemokine called human monocyte-derived neutrophil chemotactic factor CXCL8

decades. C-X-C and C families are mainly active towards neutrophils and lymphocytes, while C-C family members are active towards macrophages, lymphocytes, basophils and eosinophils [106]. A number of chemokines can signal promiscuously with many or several types of receptors, while others form monogamous signaling units with one receptor type. All receptors are G-protein coupled seven-time membrane-transpassing proteins (7TM-GPCRs) – a popular kind of target for pharmaceutics – and are named according to the group of chemokines they bind to.

Chemokine receptor expression on the surface of cells can additionally be restricted to a specialized cell type or be produced by various cell types, whereas expression is definitely not limited to leukocytes. Chemokine receptors with T lymphocyte specificity could be identified. CXCR3 and CCR5 seem to mark Th1 cells, while CCR3, CCR4 and CCR8 supposedly mark Th2 cell subsets [107].

CKRs play two roles in the immune system. Many CKRs and chemokines are involved in function of secondary lymphoid organs. Lymphocytes and antigen-presenting cells are in steady directed navigation through bloodstream and lymphoid organs. Interactions of CCR7 with CCL19 and CCL21, as well as CXCR5 with CXCL13 were identified to mediate their biased migration towards secondary lymphoid organs. These interactions seem therefore to be crucial for proper functioning and structure maintenance of secondary lymphoid organs.

On the other hand, chemotaxis is a crucial factor in building an immune response and can therefore act inflammatory. Eosinophil active chemokines include CCL5/RANTES, CCL8/MCP-2, CCL7/MCP-3, CCL13/MCP-4 and CCL12/MCP-5, CCL3/MIP-1α and CCL11/eotaxin [108]. All of these chemokines cause accumulation of eosinophils in sites of allergic reactions and contribute to the course of allergic asthma.

DESCRIPTION OF CKRS WITH EMPHASIS ON THEIR ROLE IN AA

XCR1. The ligands for this chemokine receptor are XCL1/lymphotactin and XCL2/SCM-1β (single-C-motive-1 beta). Possible sources of lymphotactin are peripheral $CD8^+$ T cells and natural killer cells. XCL1/lymphotactin was early shown to have the ability to recruit T cells [109].

Patients with allergic asthma, however, showed reduced expression of XCR1 and lymphotactin on $CD4^+CD25^+$Tregs, which were associated with immune suppression, and their dysregulation with allergy. In the same study application of lymphotactin could reverse defective Treg activity [110].

CCR1. This surface molecule has been designated CD191. It binds ligands as CCL3/MIP-1α (macrophage inflammatory protein-1 alpha), CCL5/RANTES (regulated on activation normal T expressed and secreted protein), and CCL7/MCP-3 (monocyte chemoattractant protein-3). Interaction with CCL5/RANTES has been shown to be responsible for the development of allo-specific T cell responses. In association with CCL3/MIP-1α CCR1 could be shown to influence neutrophil-mediated inflammatory conditions [111].

CCR1 is expressed by airway smooth muscle cells and is increasingly expressed in asthmatics [112]. Similarly to CXCR1, it was also shown to be involved in the recruitment of mast cells to Th2 stimulated airway smooth muscles, but not in healthy individuals [113]. This receptor might therefore be involved in asthma development through airway smooth muscle cell activation.

CCR2. CD192 is the chemokine receptor for CCL2/MCP-1 (monocyte chemoattractant protein-1), CCL13/MCP-4 (monocyte chemoattractant protein-4), CCL7/MCP-3 (monocyte chemoattractant protein-3), and CCL8/MCP-2 (monocyte chemoattractant protein-2).

CCR2 activity includes chemoattraction of monocytes, activated T cells and B cells [114]. Expression was shown to be up-regulated by IL-2 and down-regulated upon the presence of several bacterial products [115, 116]. In different studies the CCR2/CCL2 axis was supposed to be involved in asthma development because it

might also be responsible for the recruitment of immature mast cells to the lungs [117].

CCR3. This receptor has been designated CD193. It is a chemokine receptor for CCL11/eotaxin-1, CCL26/eotaxin-3, and CCL24/eotaxin-2, CCL7/MCP-3, CCL5/RANTES CCL15/HCC-2 (hemofiltrate CC chemokine-2), and CCL16/HCC-4 (hemofiltrate CC chemokine-4).

CCR3 is a highly expressed receptor on eosinophils and basophils [118]. As receptor for molecules of the eotaxin family it was shown to be crucial in the recruitment of eosinophils in an acute allergic asthmatic response.

CCR4. CD194 has two known ligands: CCL17/TARC (thymus and activation-related chemokine) and CCL22/MDC (macrophage derived chemokine).

Expression of this receptor has been associated with homing to non-lymphoid tissues. $CD4^+$ T cells in the lungs of asthmatic patients showed expression of CCR4 [119], amongst others. Interestingly, CCR4 is also expressed by Tregs counteracting the asthma phenotype [120].

CCR5. This receptor, designated CD195, binds CCL8/MCP-2, CCL4/MIP-1β (macrophage inflammatory protein-1 beta), CCL3/MIP-1αS (macrophage inflammatory protein-1 alpha S), CCL3LI MIP-1αP (macrophage inflammatory protein-1 alpha P), and CCL5/RANTES.

CCR5 expression was shown to be up-regulated by activated lymphocytes in mice challenged with aerosolized OVA [121], and might therefore play a role in asthma development.

CCR6. The ligand for this monogamous receptor is CCL20/MIP-3α (macrophage inflammatory protein-3 alpha).

CCR6 was shown to be elevated in $CD4^+$ T cells in lung tissue compared to peripheral blood of asthmatics, also in comparison with $CD8^+$ T cells [122]. This CKR is furthermore considered to be responsible for the migration and positioning of Th17 cells [123]. Even though associated to homing to intestines, it is still unclear if this receptor plays a role in the exacerbation of AA.

CCR7. CCL19/ELC (Epstein-Barr virus-induced receptor ligand chemokine), as well as CCL21/SLC (secondary lymphoid tissue chemokine) are both ligands for CCR7/BLR2/EBI1, the expression of which can be induced on B and T cells by the Epstein-Barr virus. The orchestration of these two chemokines was identified as essential elements of microenvironment establishment for proper immune response initiation in secondary lymphoid tissues [124] and to be crucial for cell trafficking and structural organization of lymphoid tissues. ELC is expressed in lymphoid tissues and activated B and T lymphocytes. It plays a role in the migration of memory T cells to inflamed tissues and is able to stimulate the maturation of DCs. SLC may play a role in homing of lymphocytes to secondary lymphoid tissue. In vitro, however, this protein was shown to be exclusively chemotactic for thymocytes and activated T cells.

CCR7 signaling was explicitly made responsible for T cell retention in T cell areas of secondary lymphoid tissues. Therefore, CCR7 signaling counteracts the signaling of CXCR5 [125] that retends cells to B cell containing follicles. In a study, ELC and SLC seemed to act as regulatory chemokines for the control of airway allergic disease [126].

CCR8. This receptor forms a monogamous unit with the inflammatory chemokine CCL1/I-309.

CCR8 is preferentially expressed on Th2 cells and is assumed to be responsible for antigen-specific Th2 cell recruitment into the lungs, and therefore, for the development of allergic asthma as well [127].

CCR9. CDw199 and CCL25/TECK (thymus expressed chemokine) form a monogamous receptor ligand unit.

The receptor is selectively expressed in the thymus and small intestines, the two known sites of T lymphopoesis. This receptor has been strongly associated to recruitment of T cells to mucosa in different ways [128]. A study of *Uehara et al.* showed that all CCR9$^+$ T lymphocyte subsets respond to CCL25/TECK and suggests that CCR9 may play an important role in the development and trafficking of both αβTCR$^+$ and γδTCR$^+$ T cells [129]. An implication of CCR9 in the development of asthma is a specific population of natural killer T cells that is recruited into the lung

with the involvement of CCR9, where they contact and stimulate Th2 cells to cytokine secretion [130].

CCR10. This receptor responds to the binding of CCL27/CTACK (cutaneous T cell attracting factor) and CCL28/MEC (mucosae-associated epithelial chemokine).

CCL27 is expressed by epithelial keratinocytes in skin inflammation and attracts $CCR10^{+}$ and $CCR4^{+}$ cell into inflamed sites of the skin and may participate in the pathogenesis of atopic dermatitis [131].

CXCR1. CD128a serves as receptor for CXCL8/IL-8 (interleukin-8), CXCL6/GCP-2 (granulocyte chemoattractant protein-2), and CXCL7/NAP-2 (neutrophil activating protein-2).

CXCR1 and CXCL8/IL-8 expressions were shown to be up-regulated in asthmatic patients and seem to be connected to neutrophilia during severe exacerbations of asthma [132]. Similarly to CCR1, it was also shown in asthmatics to be involved in the recruitment of mast cells to Th2-stimulated airway smooth muscles [113].

CxCR2. CDw128 binds, in addition to all CXCR1 ligands also CXCL5/ENA-78 (epithelial cell-derived neutrophils activating factor-78), CXCL1/GROα (growth related oncogene alpha), CXCL2/GROβ (growth related oncogene beta), and CXCL3/GROγ (growth related oncogene gamma).

This receptor was associated with the recruitment of neutrophils, especially in the development of lyme arthritis and carditis. Both protein expressions of CXCR1 and CXCR2 are also up-regulated in asthmatic patients and seem to be rather connected to eosinophilia than neutrophilia [133]. After LPS inhalation, however, CXCL5 revealed to mediate neutrophil recruitment to the lungs [134].

CXCR3. There are two types of this receptor CXCR3A and CXCR3B binding CXCL10 IP-10 IFN-inducible protein 10, CXCL9/Mig (monokine induced by gamma interferon) and CXCL11/I-TAC (IFN-inducible T cell a chemoattractant), whereas CXCR3B also binds CXCL4/PF4 (platelet factor-4).

The recruitment of Th1 cells upon Ag exposure has been associated with the functionality of CXCR3 [135]. Interestingly, increased CXCR3 expression of memory T helper cells could be correlated to increased severity of allergic asthma [136].

CXCR4. This chemokine receptor was shown to bind CXCL12/SDF-1αβ (stromal cell derived factor 1αβ) and to be a co-stimulation factor in the process of thymic β-selection [137].

Decreased CXCR4 expression by budesonide application has been shown to reduce airway remodeling [138]. Since CXCL12/SDF-1αβ was shown to control migration of endothelial cells, CXCR4-CXCL12/SDF-1αβ interaction is supposed to be involved in angiogenesis [139].

CxCR5. CXCR5 and CXCL13/BCL-1 (B-cell activating chemokine-1) are a monogamous signaling unit restricted to mature B cells [140].

CXCR5 has not been put in connection with any pulmonary irregularities so far. The receptor has been continuously brought in relation to T cell homing to areas of the lymphoid tissue and its role in proper T cell-B cell interaction, causing T cells to enter B cell follicles, while the counteracting signal of CCR7 induces their retention in the T cell area [141].

CXCR6. Functionally CXCR6 serves for cell adhesion to CXCL16/Zmynd15 (zinc finger, MYND-type containing-15) that is considered as a scavenger receptor for phosphatidylserine and oxidized low-density lipoproteins and therefore involved in an interesting signal transduction pathway. CXCR6 was shown to be expressed on the surface of T cells and NKT cells [142].

CXCR6 acquires its physiological significance during immune responses by selective up-regulation on DCs, when the interaction between lymphocytes and cells of the innate system is promoted. Indications show that CXCR6 might be implicated in the positioning and activation of T cells [143].

CXCR7. This receptor was shown to bind CXCL12/SDF-1αβ and CXCL11/I-TAC.

CXCR7 signaling was shown to be involved in T cell migration. Interestingly, this alternative CXCL12/SDF-1αβ receptor signals via a non-classical signaling pathway [144].

CX_3CR1. The fractaline receptor CX_3CR1 ligand CX_3CL1/fractaline can be expressed as a smaller soluble chemokine but also as a membrane-bound molecule with a mucin-like domain [145].

CX_3CR1attracts and activates T cells, NK cells and APCs. CX_3CL1/fractaline is supposed to mediate attachment to lung vascular epithelium, as well as, tissue invasion. In inflammatory lung diseases like chronic obstructive pulmonary disease it promotes therefore structural destruction and remodeling of inflamed tissue.

AIM OF THE WORK

Depending on their expression, chemokines are characterized as homeostatic and inflammatory or constitutive and inducible. Chemokines controlling cell homing and recirculation from inflammation sites contribute to homeostatic properties of cells. On the other hand, inflammatory chemokines are produced in response to inflammatory stimuli, to recruit cells to inflammation sites. Consequently, ***the expression pattern of chemokine receptors on a cell surface can specifically determine cell types and cell states*** because the receptor's presence dictates the range of a cell's interactions mediated by chemokines in its environment. Therefore, a basic assumption in this study is that the presence of allergen-specific memory cells in asthmatic mice – even in the absence of allergen – might influence the gene

expression of chemokine receptors in the lung tissue that are responsible for memory cell retention.

Allergen-specific memory Th2 cells are localized in the lungs of asthmatics for a lifetime [6]. As a consequence, these cells are supposed to have a migratory bias to the lungs, where they were generated. Conceivably for the same reason, localization of memory cells might be performed with the help of cell type-specific chemokine-chemokine receptor mechanisms. A major focus of this thesis will be laid on allergen-specific characterizations and cell localization during quiescent states of AA.

The second aim of this study is to expand focus on this migratory chemoattraction system to phases of asthma during the targeted Th2 cell activation. A limited number of memory cells are obtained in the recovered tissue building up the IM of an immunized organism. This IM is exclusively composed of lymphocytes encircled in populations of other resident leukocyte types that are all together embedded in an intact network of structural cells with different functions and complete attachment to flexible fibers of the extracellular matrix. In the case of asthma the retention of memory Th2 cells within cell infiltrates in the lung is strongly associated with the perpetuation of asthma [6]. Therefore, we will try to dissect changes in CKR expression during early hours after allergen re-challenge in a model of asthma relapse in mice.

In the interest of clinical medicine maintenance and activation of memory T cells in inflamed tissues is of great importance. Due to the migration-directive functions of CKRs, we are interested in comparing their gene expression between healthy and asthmatic mice before and during induction of an OVA-mediated allergic reaction. The mRNA contents of CKRs in the lungs at the moment of assessment will be retained from lung tissue and the relative mRNA amount of different CKRs in different phases of allergic asthma will be compared.

Animals and Materials

Animals

Mice. Female mice of the strain BALB/c were purchased from Charles River Laboratories International, Inc. (Wilmington, Massachusetts) and housed at the Veterinarian University of Vienna. They were provided OVA-free food (SSNIFF, Soest, Germany) and tap water ad libitum. All experiments complied with requirements of the Austrian Ministry of Science.

Sensitization and Challenge.

- Albumin, from chicken egg white, Sigma Aldrich Chemie GmbH, Catalog No. A5503-5G.
- Dulbecco's Phosphate Buffered Saline 1x,Invitrogen, Catalog No. 14190-094.
- Omega ultrasonic nebulizer, Kendall Aerodyne, Catalog No. 9230467.
- Ketanest S, Pfizer Corporation Austria Ges.m.b.H., Catalog No. 1-22525.
- Rompun 2%, Bayer, Catalog No. 8-14840.

Materials

Laboratory equipment.

- Cytospin 4 centrifuge, Thermo scientific Shandon, Catalog No. A78300001.
- GS-6KR centrifuge, Beckman , Catalog No. 244925.
- Centrifuge 5415C,Eppendorf, Catalog No. 105302.

ELISA for OVA-specific serum IgG1.

- ELISA plate 96 well, flat bottom, Iwaki, Catalog No. 3801-096.
- Columbus Plus, TECAN, Catalog No. F109204.
- Bovine Serum Albumin (BSA), Sigma Aldrich Chemie GmbH, Catalog No. A-2153.
- 0.5% Tween 20, Bio-Rad, Catalog No. 170-6531.
- Goat anti mouse IgG1 biotin, Southern biotechnology associates Inc., Catalog No. 74.
- Streptavidin-HRP, Southern biotechnology associates Inc., Catalog No. 64.
- BD OptEIA TMB substrate reagent set, BD Biosciences, Catalog No. 555214.
- Unimax 1010 DT, Heidolph Catalog, No. 543-12310-00.

RNA isolation.

- RNeasy Mini Kit, Qiagen, Catalog No. 74106
- Trizol Reagent, Invitrogen, Catalog No. 15596018
- Bio Photometer, Eppendorf, Catalog No. 6131

Gel electrophoresis.

- Invitrogen Agarose electrophoresis grade, Catalog No. 15510-027.
- Model 1000/500 constant voltage power supply, Bio-Rad Laboratories, Catalog No. 164-4710.
- Invitrogen 10x Blue Juice Gel loading buffer, Catalog No. 10816-05.

cDNA synthesis.

- First-Strand Synthesis System for RT-PCR, Invitrogen SuperScript, Catalog No. 11904018.
- SuperScript III First-Strand Synthesis SuperMix for RT-PCR, Invitrogen, Catalog No. 11904018.
- Thermomixer comfort, Eppendorf, Catalog No. 5355 000.011.

Quantitative PCR.

- Power SYBR Green PCCR Master Mix, Applied Biosystems, Catalog No. 4367659
- 25nmol Oligomers, Invitrogen
- StepOnePlus Real-time PCR System, Applied Biosystems, Catalog No. 4376599.
- MicroAmp Fast 96 well reaction plate 0.1ml, Applied Biosystems, Catalog No. 4346907.
- Optical Adhesive covers, Applied Biosystems, Catalog No. 43600954.

Analytic software.

- StepOne Software v2.1., Applied Biosystems.
- Prism 5.2., GraphPad Software, Inc.
- Microsoft Office Excel, Microsoft Corp.

METHODS

Laboratory equipment. Equipment from the Experimental Allergy laboratory was used in experiments as needed and required. DNA and RNase-free working spaces were arranged and regularly treated with 70% ethanol and RNaseZap (Ambion, Catalog No. AM9780), to prevent contamination and uphold high quality of used templates and reagents. Ethidum bromide working spaces were assigned and appropriate waste disposal set up adhering General Laboratory Standard operating procedures and Rules.

Sensitization and Challenge. Established sensitization and challenge protocols for asthma mice were supplied with courtesy by the Experimental Allergy Laboratory and were accurately followed for all animals of the test groups. The protocol of asthma model establishment in mice includes two sensitization steps, and subsequent 4 local challenges. Sensitization was reached by intra peritoneal injection of 10μg OVA in PBS injected on day 0 and 21. OVA was introduced into the peritoneal cavity to stimulate the development of systemic allergen-specific lymphocytes and therefore sensitize treated mice for OVA. Local respiratory challenges were performed with 1% OVA-aerosol to induce acute allergic reactions and with 100μg OVA in intranasal applications for the induction of asthma relapse (referred to as re-challenge in the following). For asthma establishment on day 28 and 29, mice were challenged with aerosolized 1% OVA in PBS twice daily. OVA (100m) was dissolved in 0.1l PBS and completely aerosolized to challenge mice for 1h in the morning. Mice were then left to rest at least for 4h and were challenged again in the afternoon with the same procedure as in the morning. After the challenge procedure we expect previously formed T cells in blood or the lymphatic system to be recruited into the lung parenchyma. Detection of established allergen-specific responses are performed by evaluation of histopathologic lung section staining, and qualitative cell count assays from airway mucosa and epithelial lining. After onset of acute asthmatic disease that is following challenge, all treated mice are allowed to recover in the next 3 months, after which mice are considered as recovered from acute disease. Degree of sensitization after recovery can be traced

back by the ability of asthma relapse or quantitative serum immunoglobulin analysis, both parameters indicating the presence of OVA-specific T cells.

Re-challenge of recovered mice. To induce disease relapse, mice were i.n. re-challenged with 100µg OVA (referred to as re-challenge in the following text). In order to do the intranasal application, mice were put to sleep (Ketanest/Rompun), and droplets of OVA/PBS were delivered on their nostrils until deliberately inhaled. This way of re-challenge was chosen to guarantee uniformity of application and compliance with assessment time points.

Blood sampling. The collection of blood was performed to yield cell-free serum for the investigation of allergen-specific antibody quantity in the blood. The evaluation of antibody titers is generally a reliable indication for immunity against a specific factor. High levels of OVA-specific antibodies in the blood means that the organism is sensitized against OVA. An overall volume of 1µl serum is enough for titration by ELISA. Blood was therefore collected through sampling via any lateral tail vein. Blood was allowed to clot for 2h at room temperature. Samples were then centrifuged for 10 minutes at maximum speed to pellet any solid material and enable sera to be transferred into fresh *eppendorf* tubes. Sera were stored at -20°C until antibody titers were determined.

Serum IgG1. Extent of responsiveness of mice to the treatment with OVA was evaluated by content of OVA-specific IgG1 in serum after local respiratory tract challenge. ELISA plates were coated overnight at 4°C with 0.5µg OVA per well. Residual OVA was then removed with the help of a microplate washer (TECAN). Then, plates were blocked with 2% BSA for 2h at room temperature to reduce exposed unspecific binding sites for the antibody, and afterwards washed with 0.1% Tween 20 in PBS. Finally, serial dilution of sera was applied on plate and incubated overnight at 4°C. Wells were then washed of excessive serum by the application of the microplate washer. OVA-specific avidin-linked goat antibody was added onto OVA-coated plates and incubated for 2h at 4°C. After additional washing, streptavidin-HRP Horse Radish Peroxidase was applied on coated wells to mark bound allergen-specific antibodies from serum. Tetramethylbenzidine (TMB) substrate was added and incubated for 10min for color development before reaction

was stopped with 0.18M H2SO4. Color development depends on amount of allergen-specific antibody-bound HRP inducing concentration-dependent TMB oxidation for colorimetric antibody titer evaluation. Absorbance was measured by spectrometry at 450nm.

Organ harvesting. For RNA extraction from the lungs, we performed this procedure as fast as possible to prevent RNA degradation. After cervical dislocation, the peritoneum was opened and diaphragm pierced to make lungs collapse. Chest cavity was opened without damaging lung or heart. Lungs were pulled out by holding the trachea with a forceps and then by careful dissociation from connective tissue. Lungs were then perfused with 10ml ice-cold PBS and with additional 5ml RLT buffer supplied by QIAgen to additionally save RNA from degradation. Perfusion was performed by gaining access to the lung's arterial system with a needled syringe through the right ventricle, to remove as much blood-derived leukocytes as possible.

RNA extraction. Total RNA with lengths of more than 200bp [146] was isolated from total lungs using RNeasy Mini Kit (Qiagen, Catalog No. 74106), according to manufacturer's recommendations. Before RNA extractions, however, lungs were perfused with PBS to remove circulating leukocytes. Then, lungs were homogenized with RLT buffer to which β-Mercaptoethanol was added. Tissue was disrupted using a Rotor-Stator homogenizer. Homogenates were centrifuged and supernatants were aliquoted: one aliquot was used for RNA extraction and others were immediately frozen in liquid nitrogen and stored at -80°C.For RNA extraction, lung homogenates were mixed with ethanol to adjust binding conditions, before they were applied on silica-membranes. After binding, RNA membranes were washed with supplied ethanol containing washing buffers. Elution was performed with 80µl RNase-free water. RNA quantities between 50µg and 80µg were expected.

Spectrophotometry. Absorption at 260nm and absorbance ratios between A260nm and A280nm or A230nm were used to determine RNA extracts' concentrations and purities, respectively. Dilutions were prepared, to reach an OD-value between 0.1 and 1, to ensure appropriate measurement. We used a buffered solution for dilution (TAE buffer) and created them from samples at comparable temperatures to increase reproducibility as much as possible. After extraction, RNA concentration

evaluation is recommended by absorption of light at a wavelength of 260nm. OD 1 is equivalent to an amount of 40µg RNA. Contaminations with protein can be excluded at a A260nm/A280nm ratio of approximately 2 and above. Contaminations with salts are minor at A260nm/A230nm ratios above 1.5.

Gel electrophoresis. Electrophoresis was used to check integrity of extracted RNA molecules by separation on 1.2% formaldehyde-agarose gels under denaturating conditions. 2µg RNA was incubated with formaldehyde for 5min at 60°C to denature molecules. Gels were run for 1h at 50mV, and 18S and 28S bands were evaluated under UV-light for band sharpness. 1% agarose gels were used for determination of qPCR product length and purity. Gels were run for 45h at 65mV and evaluated under UV-light. To all gels 100µg ethidium bromide per 100ml gel was added. Ethidium bromide is a fluorescent DNA-intercalating molecule that is routinely used to make nucleic acids visible without specificity. It fluorescents light-blue in UV-light. Because of the application of UV-light and the toxic and carcinogenic agent ethidium bromide, this method requires a high degree of caution in handling and preparation.

cDNA synthesis. After RNA was extracted, purity and integrity was checked to guarantee RNA extract quality. cDNA was synthesized from extracts using oligo(dT) primers and Superscript II First Strand Synthesis Kit or SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen). Under our conditions only fully transcribed polyA-tail containing mRNA and transcription products with adenylated 3' ends after processing are used as templates to generate complementary DNA fragments without further specification for length or sequence, as long templates are longer than 200bp.

Quantitative PCR. Sequence-specific amplification of DNA is the same underlying principle of standard and quantitative PCR with the difference that the amplification during the latter is monitored in real time. The speed at which the amplification products reach a detectable level is extrapolated to determine the copy number of starting material or to evaluate relative amounts of different templates before reaction. In this study the difference of relative gene expression to HPRT expression between samples of two groups is used to interpret trends in expression regulation (delta-delta Ct-method). When more groups are evaluated, relative expression of β-

actin to HPRT was additionally measured in all groups and used as reference gene, to make statistical analysis more accurate. The evaluation of amplification kinetics is possible through the application of fluorescent DNA dyes or nucleotide oligomer probes. In this case the unspecific fluorescein-derived SYBR Green was used. Relative expression of the investigated genes was determined amplifying 1ng cDNA primed by 300nM gene specific oligomers (Invitrogen*)* using SYBR Green Master Mix (Applied Biosystems) in StepOnePlus Real-Time PCR System (Applied Biosystems). Whenever it was necessary due to low expression, primer efficiency was evaluated at higher template concentrations and cDNA concentrations of 10ng per reaction were used. In the case of TCR-vα13 expression evaluation, cDNA amount that showed primer efficiency closest to 100% was applied to adapt to low expression in whole lung. Relative expression between test groups was calculated using the Pfaffl-method. Expression difference between two groups is calculated and ratio to reference gene expression difference between the groups is evaluated considering primer efficiency [1]. To apply statistical analysis, all data of a run was calibrated to the same sample in an experimental group of three or five mice. For all experiments considering memory maintenance, data was calibrated to one of the samples from the age-matched control group. For experiments considering the acute allergic response, one of the samples from the recovered group was used as calibrator. Box and whisker plots were used to display data results illustrating median andupper and lower quartile, and maximum and minimum, respectively.

AD RELATIVE GENE-EXPRESSION

With the help of the qPCR method it is not only possible to calculate copy numbers of a RNA molecule, this method is also an important tool to determine different gene expression rates of cells. To investigate a gene product's biological significance, it is useful to know about its expression's up- or down-regulation at gene transcription level in different physiological conditions, for instance. To know that a protein is synthesized in higher rates when the surrounding environment changes, gives indications about the role it might play in the life of a cell or organism. Additionally, if a protein is steadily produced regardless of environmental conditions, it might be necessary for survival. Genes permanently expressing these proteins can be summed under 'house-keeping genes'.

What happens in the process of qPCR is the same that happens in all polymerase chain reactions: DNA is amplified. In qPCR, however, the amount of amplification product is monitored throughout the whole run after each cycle. This is possible through the use of fluorescent dyes and fluorescence detectors. In this study SYBRGreen –an intercalating derivate of fluorescein – is used as DNA dye. According to theory, amplicon• amount is doubled in each cycle. As long as there are enough reagents and active enzymes, this process continues. In the first cycles no signal will be detected because the amount of DNA is simply too low. In the end of a run the signal will reach a plateau where the amplification slows down continually.

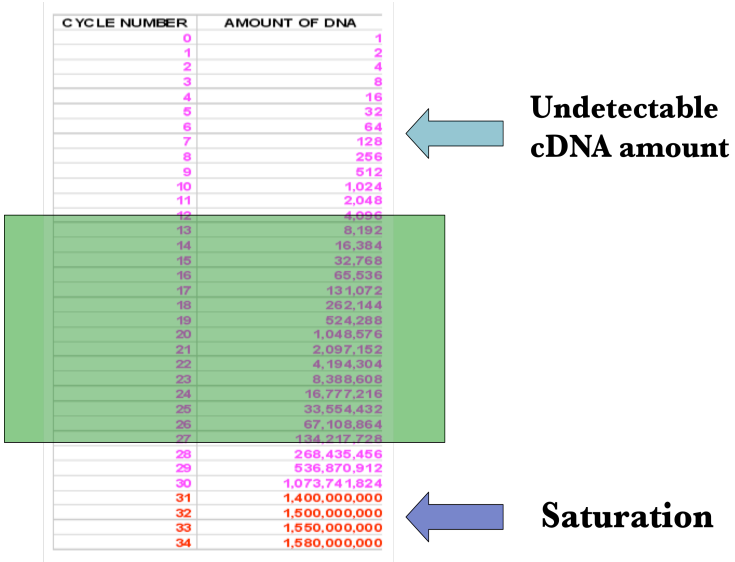


Figure 4.Theoretical exponential amplification of a cDNA template during a typical PCR reaction. Illustration by Dr. Margaret Hunt. Published at http://pathmicro.med.sc.edu/pcr/realtime-home.htm.

• comprises sequences amplified during PCR, each PCR product is identical in sequence and length

For the reason of visualization, the relative fluorescence is plotted in a semi-logarithmic diagram (next figure). There are different phases of PCRs become immediately visible. After the noise phase the signal exponentially emerges out of the background. Then the signal reaches a linear range in which the doubling of DNA correlates directly to the continuation of relapsing cycles. This linear range is the relevant phase of a qPCR run.

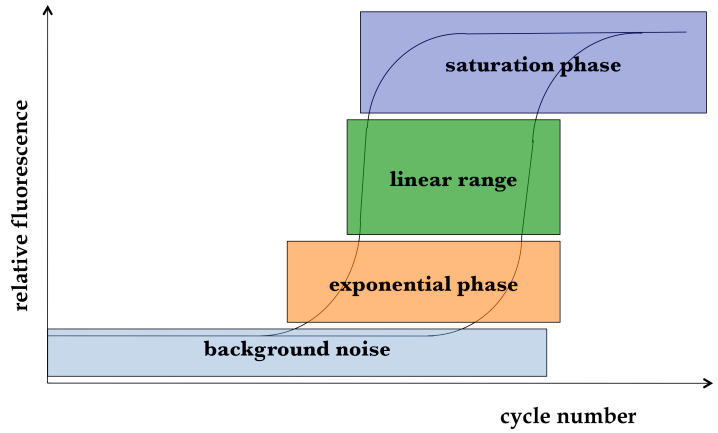


Figure 5. Schematic course of a typical qPCR assay, in which two signals are increasingly detected with time. For analysis the logarithm of relative fluorescence of a reaction is plotted against elapsed cycles. In this way of visualization the amplification of a specific DNA sequence until saturation appears as a sigmoid curve.

Contrary to most other methods, information of yielded qPCR results is extracted from a plot depicting measured fluorescence against the elapsed cycles. It is important to keep in mind that the amplification of a template depends on the amount of its starting material. The essential step in data analysis is setting the cycle, in which the signal is unmistakably detected for the first time. Logically, this cycle is reached the sooner, the more templates are available at reaction start. This quantity-dependent cycle time point makes quantification possible and can be evaluated in fractional numbers to make the assay more accurate. So, the second step in analysis is to determine a threshold for each gene and evaluate the time points, when the deliberately set fluorescence threshold on the plot is surpassed. The yielded data is termed Ct of a reaction and is consequently used further for analysis.

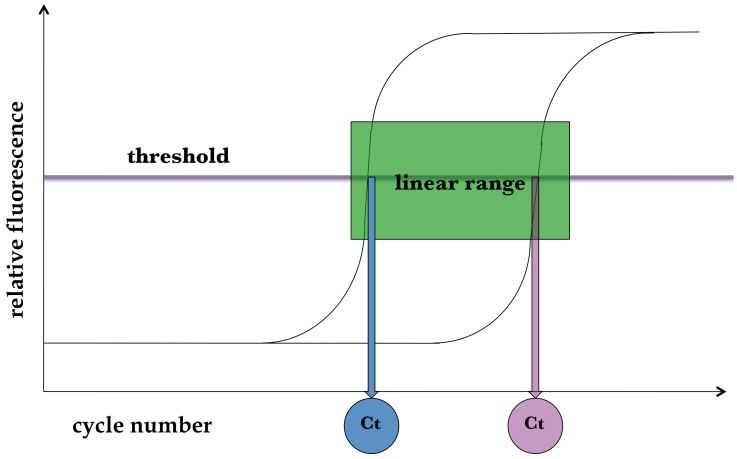


Figure 6. Detection-threshold. After each run, a specific threshold of detection is set for each gene. When the signal exceeds this threshold the yielded cycle time point is used for further analysis – termed Ct. This analysis step influences sensitivity of detection and leads to result aberration, when not performed properly. Generally, the threshold has to be set as low as possible somewhere above background and below the middle of the linear range. Thresholds should be set manually, what implies that here most variations between experimenters can occur.

DETERMINING A REFERENCE GENE

Unconditionally expressed genes are generally referred to as *house-keeping genes*. They are necessary for survival or normal functioning of every cell, either through their essential involvement in metabolism, cell-structure or replication machinery. According to definition, it does not matter in what state a cell is, these expression products have to be available at all time and their genes are therefore expressed at all time at the same rate.

Housekeeping genes and their expression rates are so precious for qPCR, since being necessary for evaluation of relative gene expression. Using their constant expression rate as a baseline the generated data of the target gene amplification can be **normalized** to it and then compared. Therefore, not the values of the target gene amplifications are compared to each other; it is their relative expression to a housekeeping gene. Normalization prevents unintended aberration of the results through irregularities during sample and reaction preparation.

To determine an appropriate *reference gene*, amplification of the candidate housekeeping gene has to be performed with every sample applying the same amount of template. Supposing that the template concentrations are not equal but comparable, the gene that is amplified throughout all the different samples with the least deviation can be used as appropriate reference gene for analysis.

Quantitative PCR is a sensitive method compared to other expression quantification techniques available at the moment. To elicit minute changes of expression, normalization and calibration have both to be done in a high degree of accuracy. The most commonly used approach to normalize expression patterns applies the use of internal controls, or in other words the use of housekeeping genes as references. They are considered as being constitutively expressed and are, therefore, defined as 'baseline expression'. It is the difference to the baseline that makes it possible to compare any gene expression between different samples. This step is termed **normalization**.

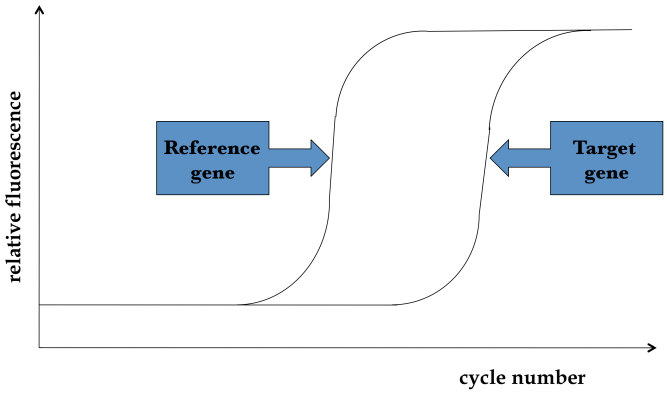


Figure 7. Normalization In qPCR, amplification of a target gene in different samples is not directly compared. Expression is first normalized to the baseline expression of a constitutively expressed reference gene. This step in analysis is referred to as normalization.

For the normalization of each assay, target gene and reference gene amplifications have to be performed in the same run and with the same master-mix. These factors are very crucial for following analysis, to make interpretations accurate. The resulting curves of these two gene amplifications are used for the comparison of expression between each target gene.

The variation between the amplification of a reference gene within different samples is ideally very low. The significance of normalization by means of house-keeping genes is not only in the realm of the sample's cell biology, it is also useful in the matter of leveling out experimental variences. If all amplifications are performed properly, any change in the target gene amplification, due to the changing amount of applied template, will be able to be observed, as well, in the house-keeping gene amplfication.

The less the deviation between house-keeping gene amplification throughout samples, the better the quality of the house-keeping gene to serve as a reference. In this study HPRT was tested and qualified best for this purpose.

PRIMER EFFICIENCY

When optimal reference gene and primer pairs for all genes are chosen, amplification efficiency of these primer pairs in the used concentration of the samples has to be determined. Therefore, amplification throughout an extended range around the used template amounts is determined and the yielded results plotted in a dilution curve.

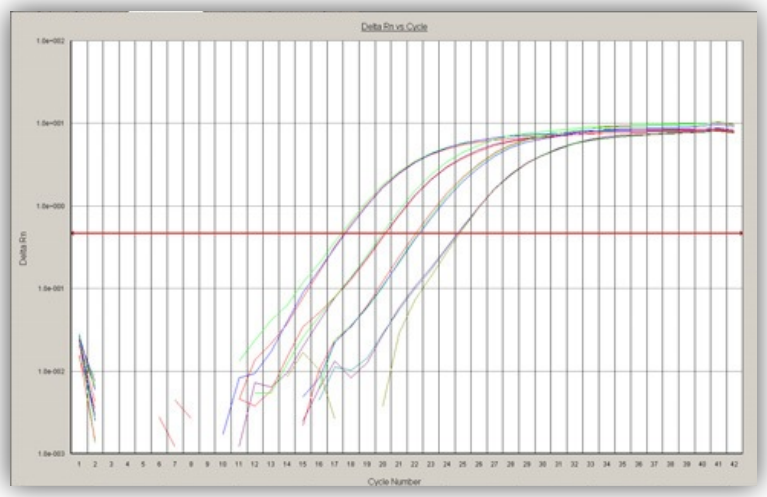


Figure 8 [1]. Results of gene-specific amplification of a serial dilution in a semi-logarithmic depiction. Amplification follows specific rules that base on the concept of standard PCRs.

All data points of this dilution curve are optimally in a regular interval of approximately 3.000 cycles. The transformation of the semi-logarithmic depiction of the measured fluorescence has implicationsthat result in different important assumptions for expression rate interpretations. The most important one is that 1000-fold expression differences will yield amplification differences of 10.000 cycles that were originally seen as proportionally increasing fluorescence intensity. Secondly, the linearized negative reciprocal value of this curve's slope will be

optimally around 2.000. This value represents the amplification efficiency and theoretically indicates the doubling of the amplification product with each cycle.

DELTA-DELTA Ct

Summa summarum the relative quantification step of this study applies the use of stably expressed HPRT that will serve in calculating the differences of target gene expression. The only parameter that influences Ct values of similar samples is supposed to be the amount of applied reagents, not the specific amount of the gene's cDNA due to different gene expression. The closer the amount of target gene mRNA to the amount of reference gene mRNA, the smaller is the difference of their Ct values. Note that the Ct value reflects the quantity of starting material of cDNA templates.

The simplest way to depict this difference of reaction starting template amounts, is achieved via delta Ct values (dCt), the value that is calculated subtracting the reference gene's Ct from that of the target gene. This operation is performed because the resulting value is theoretically reproducible with the same sample in different reactions, regardless of fluctuations in reaction preparation, because of two reasons. Primarily, since template concentration of every gene is constant in a RNA sample, variations between similar samples are leveled out for each reaction preparation. Secondly, supposing the template amount is equal, the only parameter affecting the target gene's Ct value of different samples is their extent in gene expression. Now it should become clear how the relative quantification is possible further on. The dCt value will change according to the change of the target gene expression.•

• while reference gene expression stays constant

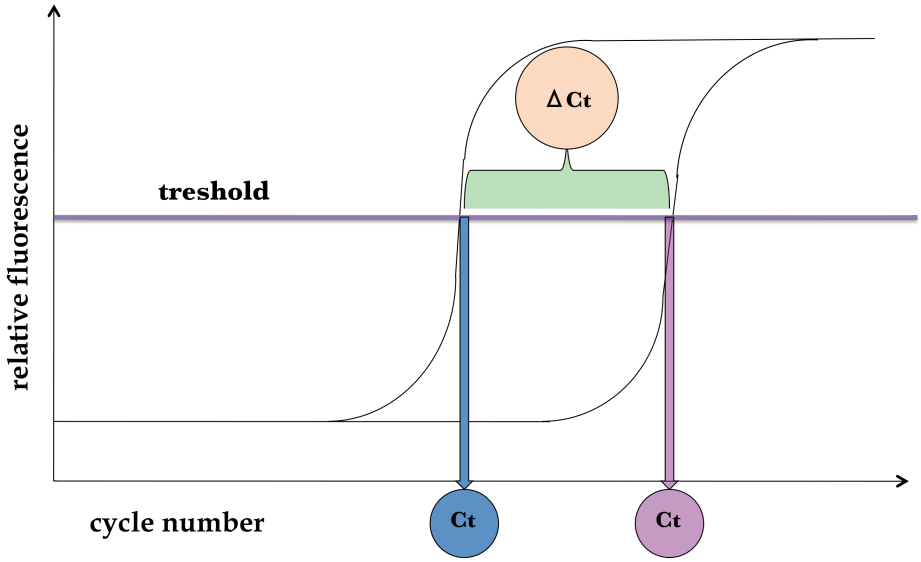


Figure 9. Here is the depiction of a qPCR run of two different genes using the same sample. Depending on the amount of starting material Ct values of the different gene product amplification is low – when a lot template is available – or high – when template expression is low. Difference of Ct values increase with increasing expression differences between genes. Evaluation of the dCt between reference and target gene is referred to as normalization.

Calculation of dCts leads to the acquisition of interpretable data. For the sake of depiction and comparability between different runs, one of these values will be set to zero. This operation will allow us to interpret the date using all available data without pooling, in order to broaden our possibility for statistical analysis. This value is called **calibrator** and can be any value of the samples, preferably one of the control groups that is close to the geometric mean within the group. Each sample including the calibrator itself has to be depicted relative to the calibrator. The necessary operation in this case is the subtraction of the calibrator's value from the values of the other samples.

The resulting value is called 'delta-delta Ct' and its negative value indicates the alteration of gene expression.

This analytical method implicates optimal amplification efficiency. Since the minute changes between our experimental groups could be masked by this misconception, we decided to use the Pfaffl-method that represents a more realistic approximation to measured values than the delta-delta-Ct method. In this operation each primer pair efficiency is used to linearize the expression difference of a gene. Then ratio between this value of target and reference gene is used to evaluate gene expression fold difference between two groups. Finally, $\log_2$ of expression fold differences are use for depiction, to make expression trends more visible.

Data analysis. In addition to above-mentioned analysis software, evaluation of qPCR data required mathematical and statistical operations. For analysis of qPCR data the Pfaffl-method was applied. HPRT was used as reference gene, since normalization shows high sensitivity for low concentrated samples [147]. The yielded $\log_2$[gene expression fold changes] were used for result depiction as boxes and whiskers.

Statistical analysis. Recovered and age-matched control groups were all compared by unpaired Student's t-test. Experiments investigating the acute asthmatic response – with eight samples per gene evaluation – were analyzed by two-way ANOVA to study the statistical differences among the four re-challenged groups to their baseline expression of β-actin. Values of $p < 0.05$ are considered significant for all evaluations.

RESULTS

DIFFERENTIAL GENE EXPRESSION OF ASTHMATIC MICE DURING RECOVERY FROM ACUTE DISEASE

Effects of sensitization with OVA on blood serum of recovered mice

To install asthma in healthy mice, every experimental animal had to undergo a time- and concentration-dependent treatment with OVA. Therefore, we treated every mouse according to the protocol of asthma establishment in mice – as described in the method section – to generate mice that are sensitized to OVA, locally challenged to induce allergic asthma and have recovered from acute disease. After the last treatment, blood samples were taken to yield antibody-containing sera that were used for ELISA-plate immuno-blots to test OVA-specific IgG1 titers. IgG1 secretion is the consequence of endocrine Th2 influence on B cells that induces Ig-class switching and cause elevated sensitizing allergen-specific antibody production. Asthmatic mice show elevated OVA-specific IgG1 levels, a reflection of helper cell-mediated sensitization, after treatment with OVA (Figure 10). Mice with *high* IgG1 titers were grouped together as recovered asthmatic mice used for following allergen-specific memory maintenance or acute asthma relapse experiments.

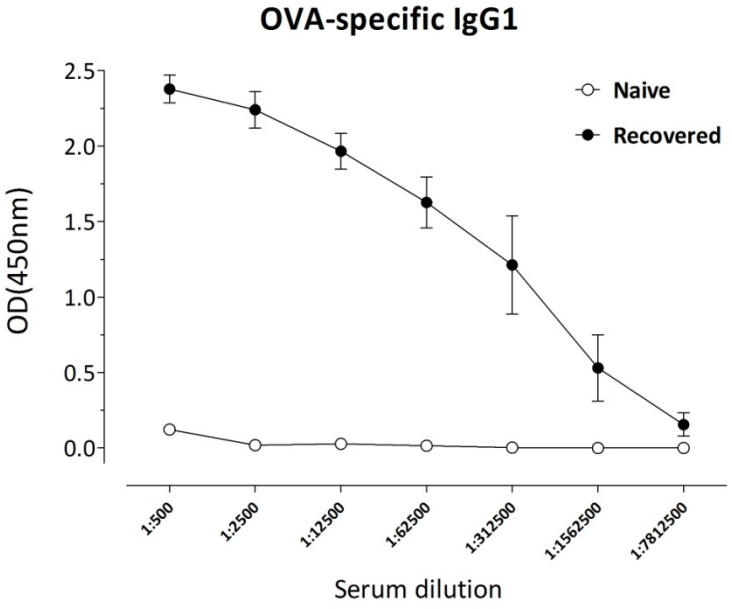


Figure 10. OVA-specific antibody titers of healthy mice, and mice after recovery from treatment with OVA. Detectable HRP activity in serum dilutions of approximately 1:7,500,000 were considered as a signal for high IgG1 OVA-specific antibody level containing serum.

Expression of common T cell markers and vα13⁺ TCR variants during recovery of asthmatic mice

We assume that, after mice were given 3 months of recovery from acute disease, differences between the recovered and the untreated mice in lung tissue gene expression will result from any allergen-specific modulation of the immune system. Therefore we hypothesize that alterations of adaptive immunity-related gene expression in mice recovered from acute disease will be a result of resident cells that cause the perpetuation of AA. To investigate this, we examined the relative gene expression of known Th2 cell-specific marker genes, IL-4, IL-5, IL-13, CD4, GATA3, CRTh2, ST2, and IL-25R, between healthy and asthmatic mice. None of the examined genes showed differently regulated gene expression between the two groups (Figure 11).

As mentioned in the introduction, there exist immunodominant domains in antigens that are preferably produced in the process of antigen processing. These peptides are preferentially bound by specific MHC molecules and recognized by specific variants of TCRs. One immunodominant OVA peptide is epitope 323 – 339 that preferentially binds to MHC class II molecule I-A^d and recognized by TCRs containing variable sub-chain types α13 and β8.1/8.2 upon antigen-presentation [148]. We decided to monitor the increase of OVA-specific immunity via TCRv-α13⁺ gene expression (Figure 12) using a forward primer that is specific for the Joining-domain αD0 and a reverse primer complementary to a TCRv-α13 coding sequence, commonly used for genotyping [149] of DO11.10 mice•. There is an average 1.59-fold increase in TCRv-α13 gene expression in asthmatic mice compared to naïve mice. This increase might indicate the increase of OVA-specific immunity in OVA-sensitive asthmatic mice.

•DO.11.10 mice are transgenic animals that express only the variable chain variant α13

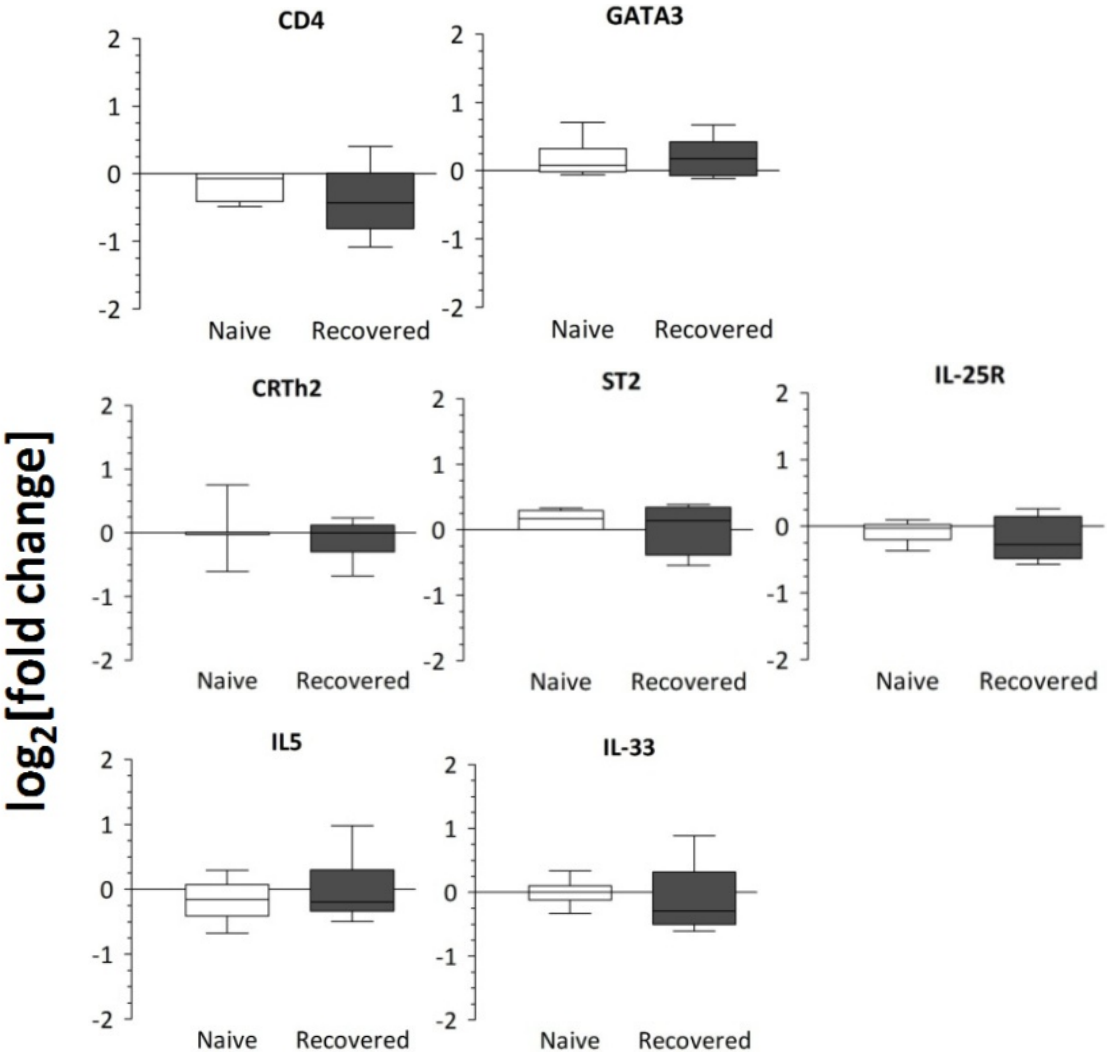


Figure 11. Relative expression of common Th2 cell markers, in naïve mice compared to mice after recovery from an acute asthmatic attack, depicted as $\log_2$[expression fold-change]. Healthy untreated mice are used as naïve age-matched control group, while the test group consists of OVA-sensitized mice, 3 months after being locally challenged with 1% OVA-PBS aerosol. All groups consist of at least 4 to 9 mice and technical replications of the runs were pooled and plotted with mean (horizontal middle line), extremes (whiskers) and $Q_{.5}$Quantil (box).

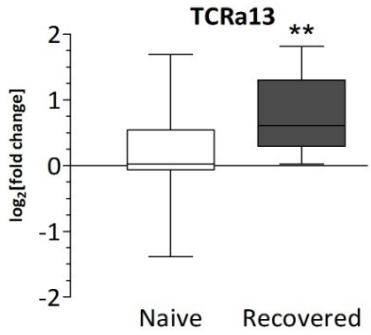


Figure 12. Relative expression of T cell receptor gene variant α13,correlated to OVA-specific immunity, as $\log_2$[expression fold-change]. Untreated mice are compared to OVA-sensitized mice, 3 months after being locally challenged with 1% OVA-PBS aerosol. All groups consist of 12 mice and technical replications of the runs were pooled and plotted with mean (horizontal middle line), extremes (whiskers) and $Q_{.5}$Quantil (box). **p-value < 0.001

Pulmonary expression of CCR6 and CCR9 is differently regulated in asthmatic mice during recovery phase

Expression of CKR genes in allergen-specific memory Th2 cells might be specific in the lungs of asthmatic mice. Concerning immunity-related gene expression in mice recovered from acute disease, we expect no alterations except from genes that are involved in the maintenance of the allergen-specific memory phenotype.

After the treatment according to the previously introduced protocol for the establishment of memory mice, the presence of OVA-specific memory cells is assumed to be the only difference between the test group and the control group. To investigate the influence of the presence of OVA-specific memory cells on the chemokine system in lungs of asthmatic mice, CKR-mRNA levels in the lungs of naïve and recovered mice were compared. In the whole lung CCR9 (1.51-fold) shows the only significant up-regulation between healthy and asthmatic mice (Figure 13A). Beside CCR9, only CXCR3 shows a robust trend towards increased expression in recovered mice (1.21-fold; Figure 13C). In contrast, only CCR6 (-1.18-fold) is significantly down-regulated in the recovered phase in comparison to mice in healthy state (Figure 13A). CCR1 is the only other gene that shows a similar reproducible down-regulation trend (-1.34-fold; Figure 13B). All 4 genes reached reproducible but only slight expression alteration trends between the healthy and asthmatic group. None of the other known CKR genes showed statistically significant expression changes.

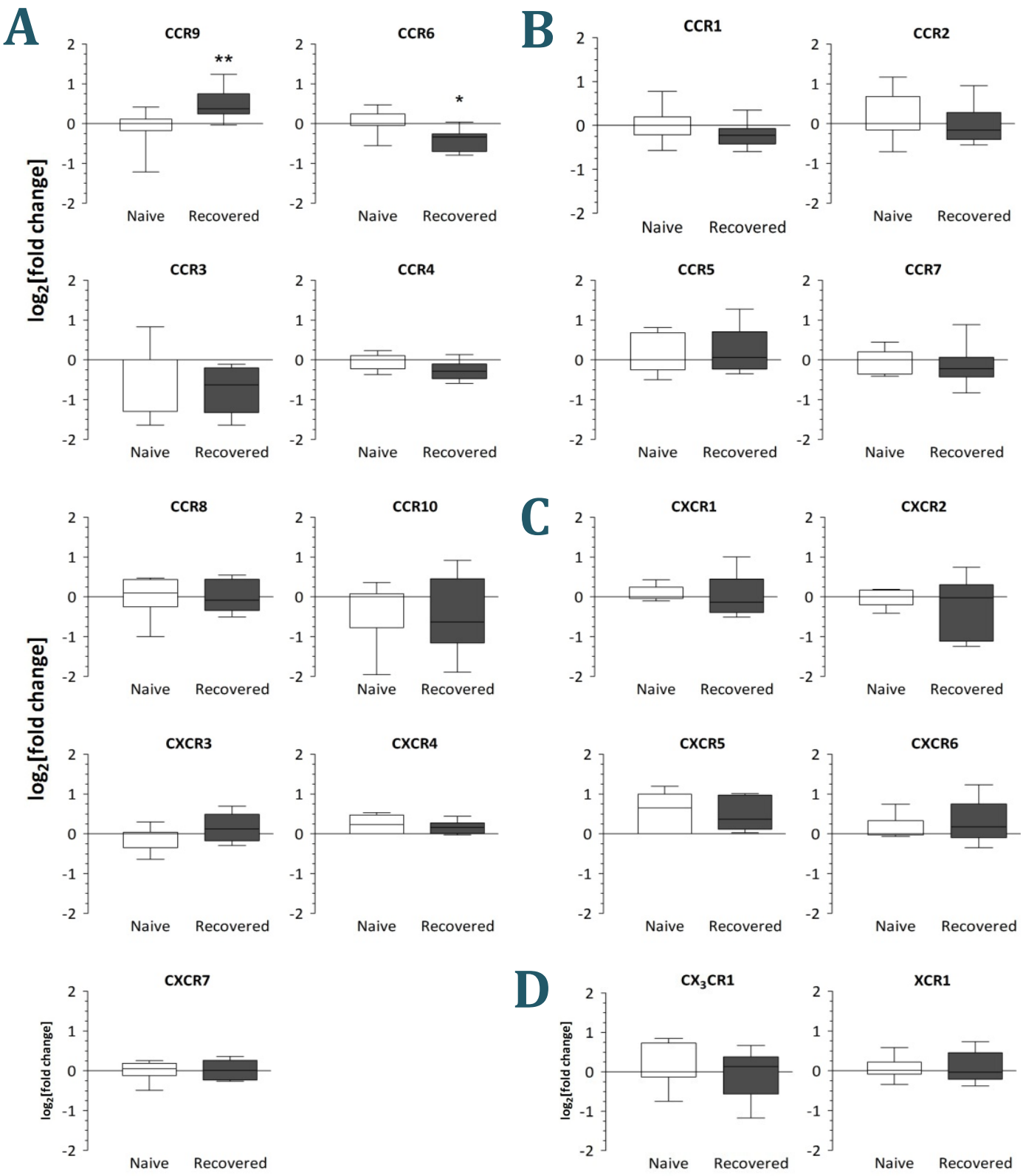


Figure 13. Relative expression of known (A) differently and (B) not differently regulated CCR genes, (C) known CXCR genes, (D) XCR1 and CX_3CR1 in healthy mice compared to mice after recovery from an acute asthmatic attack. Expression of HPRT is used as a reference gene expression and plots are depicted as $\log_2$[expression fold-change]. Healthy untreated mice are used as naïve age-matched control group, while the test group consists of OVA-sensitized mice, 3 months after being locally challenged with 1% OVA-PBS aerosol. All groups consist of at least 4 to 9 mice and technical replications of the runs were pooled and plotted with mean (horizontal middle line), extremes (whiskers) and $Q._5$Quantil (box). *p-value < 0.05, **p-value < 0.001

DIFFERENTIAL GENE EXPRESSION OF ASTHMATIC MICE DURING ACUTE ASTHMA RELAPSE

Strong up-regulation of IL-4 indicates a helper type 2 immune response in lungs of Th2 memory mice, after administration of OVA.

To get insight into early molecular events upon memory Th2 cell activation, sensitized mice were challenged, left for recovery to generate asthmatic mice and re-challenged i.n. with 100µg OVA. Eight mice per group at least were assessed at 2, 4, 6, or 14 h after challenge. Gene expression was then evaluated and compared before and after allergen challenge at different time points, using HPRT as a reference gene.

We know from previous qPCR data that IL-4 is an appropriate control to compare the behavior of the samples from the re-challenged groups regarding their correlation to a Th2 type response. Every mouse displaying a high OVA-specific IgG1 titer is expected to highly express IL-4 upon exposure to allergen because activated mast cells and Th2 cells express it. After OVA-specific IgG1 production, we examined relative IL-4 mRNA expression of asthmatic mice, before we include them to following gene expression evaluation of asthma relapse experiments. All re-challenged animals showed an immense (min. 14.000-fold) and stable up-regulation of IL-4 mRNA expression in their whole lungs following i.n. administration with OVA (Figure 14 B).

As indicated, gene expression was determined in relation to the gene expression of HPRT. This technical operation sets the baseline HPRT expression to a relative expression of 0, which does not reflect a physiological expression baseline. Thereupon, we defined β-actin gene expression relative to HPRT expression as a baseline and used it as reference gene expression for statistical analysis.β-actin gene expression relative to GAPDH was used to test this baseline expression. As expected, expression of β-actin was not differently regulated after re-challenge, (Figure 14A), and seemed therefore to be a good reference for statistical analysis.

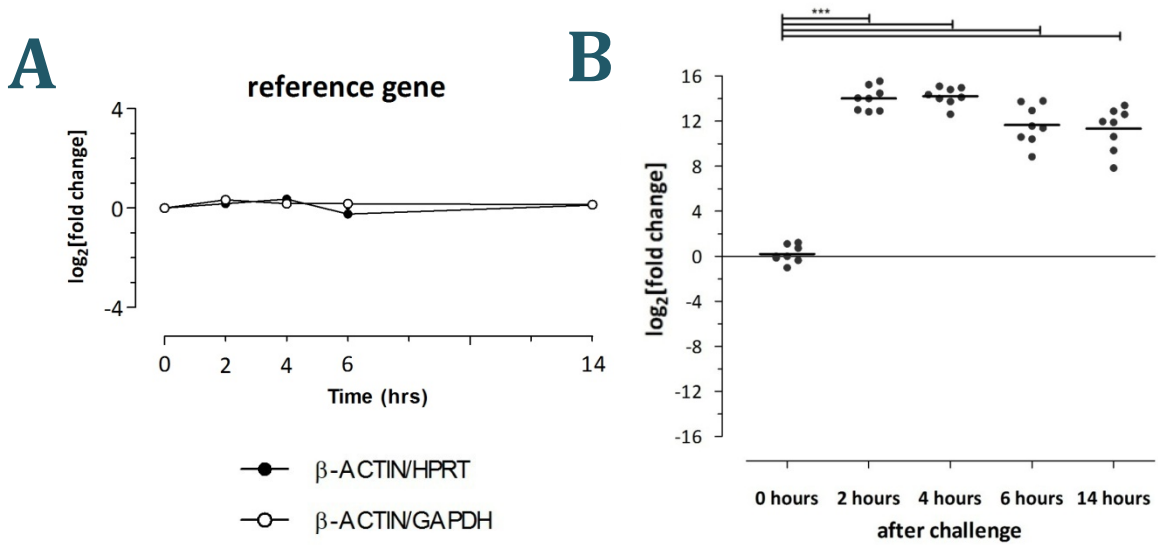


Figure 14. IL-4 expression is used to monitor Th2 type response after re-challenge with 100µg OVA i.n. All samples from challenged animals are used to evaluate IL-4 expression to control asthmatic reaction (B). Mice that do not express IL-4 upon exposure to OVA or do not behave similar to other mice in the group should be excluded from analysis. Aberrant expression of IL-4 might indicate that the mouse did not respond, i.e. to the sensitization process, and can therefore not be grouped to asthmatic mice. To enable statistical analysis and determine expression differences to baseline, expression of β-actin relative to HPRT (A) was evaluated after re-challenge and confirmed to be uniform in relation to GAPDH expression.

Time dependent IL-4, IL-5 and IL-13 expression after local antigen challenge, is OVA-specific and indicates Th2 type response.

Depending on the cytokine milieu, naïve $CD4^+$ cells polarize into effector T helper cells during priming events via interaction with peptide bound $MHCII^+$ DCs. In sensitized individuals, bias into Th2 and B cell-mediated immunity is induced and controlled by the presence of T helper cell cytokines IL-4, IL-5 and IL-13 [150]. The release of especially these three interleukins, amongst others, is characteristic for an allergic asthmatic reaction, however, little is known about their kinetics. Therefore, we examined the expression of these interleukins at different time points of asthma relapse and compared them to INF-γ expression to determine the creation of a T helper cell differentiation and activation bias. This bias is created by the release of specific cytokines leaving a cytokine expression signature with interleukin concentrations in different levels along the chosen time points.

All known Th2 cytokines are immediately, extensively and permanently up-regulated after allergen challenge (Figure 15 A). When compared to expression of β-actin with two-way ANOVA all Th2 cytokines are significantly up-regulated at all

time points after re-challenge. IFN-γ is significantly up-regulated at the 4 and 6 h time points after re-challenge. According to our data, a helper cell differentiation and activation bias built up by diverse cytokine expression profile has clearly been established at the 2 h time point and might be used to create the environment for an immediate type 2 skewing adapted response to the exposure with allergen.

Whether this response is allergen-specific or not can only be answered, if sensitized mice are re-challenged with a peptide they are naïve to. To test, if this immense up-regulation of Th2 cytokines is caused by the exposure to OVA, we challenged recovered mice with 100µg BSA i.n. and evaluated Th2 and Th1 cytokine expression in comparison to expression of β-actin. Cytokine expression pattern was clearly different in BSA challenged mice at 2 h after re-challenge (Figure 15 B). While IL-4 expression was not increased in BSA challenged OVA sensitized mice, IL-5, IL-13 and IFNγ expression showed additional signs of being down-regulated at 2 h after antigen exposure. Our data provide a strong evidence that treated mice incorporate OVA-specific lymphocytes that strongly up-regulate Th2 cytokine expression upon allergen exposure with high specificity.

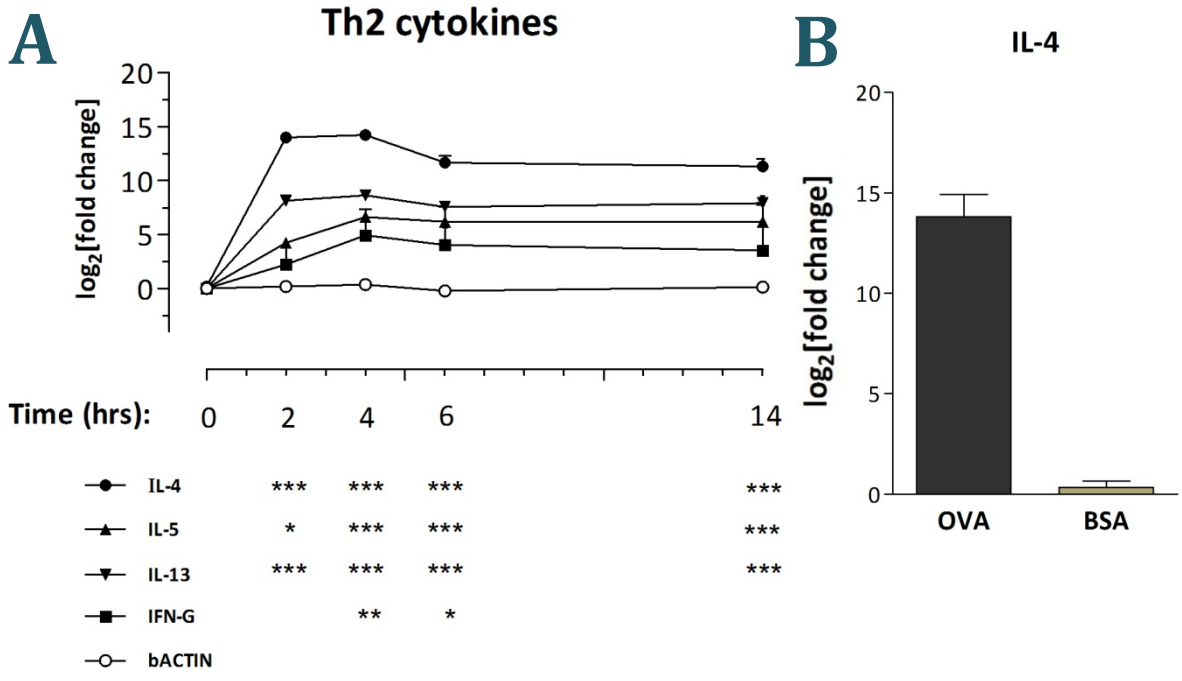


Figure 15. Expression of T helper cell cytokines were monitored after allergen challenge in sensitized mice (A) and show immediate up-regulation with a maximum at 2 to 4 h. Th2 cytokine expression stays at high levels even after the 14 h time point. When challenged with BSA instead of OVA (B), asthmatic mice show lower cytokine expression levels after 2 h.

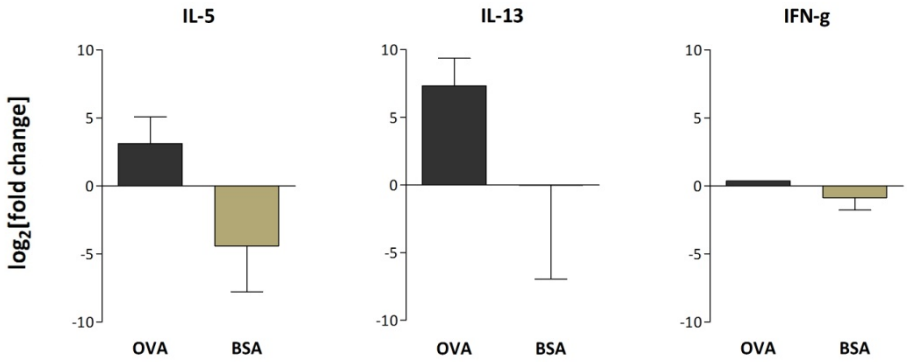


Figure 15. Expression of T helper cell cytokines were monitored after allergen challenge in sensitized mice (A) and show immediate up-regulation with a maximum at 2 to 4 h. Th2 cytokine expression stays at high levels even after the 14 h time point. When challenged with BSA instead of OVA (B), asthmatic mice show lower cytokine expression levels after 2 h.

Expression of secreted asthma promoting factors is immediately up-regulated, especially from 2 hours onwards, after allergen challenge.

The presence of allergen in the lungs of sensitized mice triggers a type 2 specific immune response that immediately sets off the release of inflammation mediators, and induces the surrounding lung tissue to the production of chemotactic and signaling peptides and their release into the lung interstitium. We tried to examine this immediate tissue response to granulocyte-derived factors by comparison of differential mRNA expression of several asthma-promoting factors after allergen exposure.

We evaluated the expression of eosinophil chemotaxis inducing chemokines eotaxin-1 and eotaxin-2. Expression of these eosinophil attracting signal molecules was immediately significantly up-regulated after allergen exposure, when compared to β-actin expression, and was maintained at high levels throughout the first 14 h of acute asthma relapse (Figure 16).

Exposure of IL-33 to lungs of mice was shown to promote the complete asthma phenotype through signaling via its receptor ST2, to induce Th2-associated cytokines, and was therefore evaluated to monitor T helper cell activation. IL-33 expression is significantly up-regulated after re-challenge, when compared to β-actin expression (Figure 16). TSLP expression was shown to promote the maturation of DCs and will indicate when antigen-presentation is actively induced. When compared to β-actin expression, TSLP was significantly up-regulated at the 4 and 6 h time points (Figure 16).

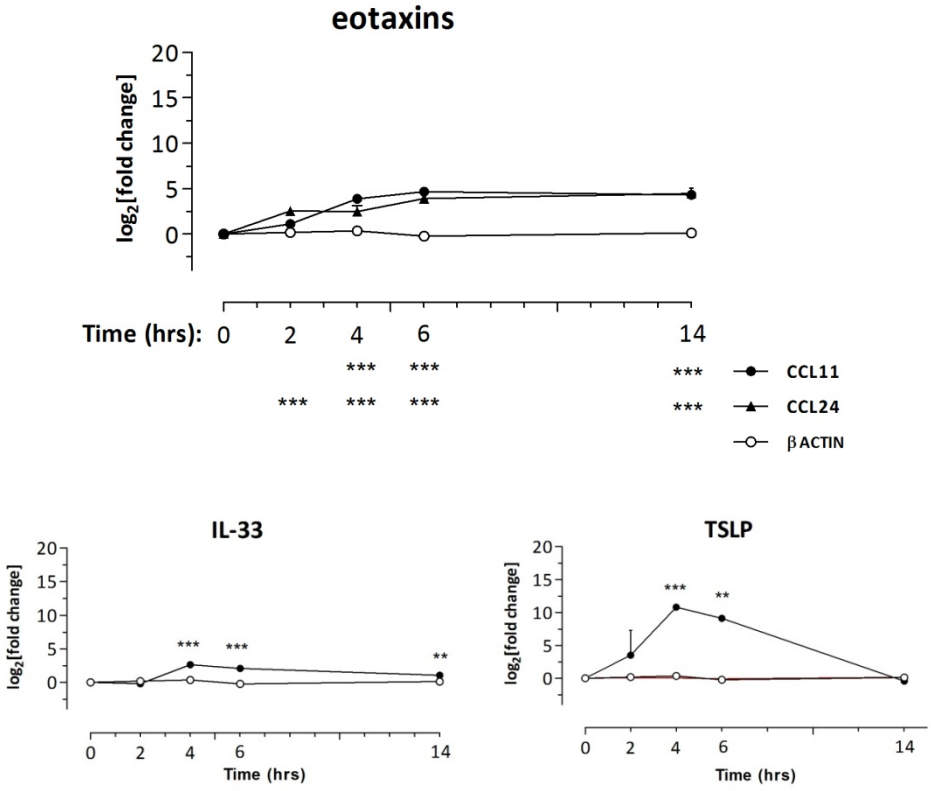


Figure 16. Expression of secreted asthma promoting factors is immediately up-regulated after allergen challenge. Expression of secreted factors associated to type 2 responses were evaluated and monitored during the early course of an asthmatic relapse. Eotaxin-1 and eotaxin-2 are shown to be similarly up-regulated to an approximately constant 16-fold increased expression change, while IL-33 and TSLP expression are up-regulated after 2 h after re-challenge, peak at 4 h, and show progressing decrease of expression towards normal levels around 14 h after challenge.

Over-all T helper cell TCR complex-associated membrane protein expression in the whole lung is equal before and after allergen exposure in the lungs of asthmatics.

Cell number dynamics indicate either cell proliferation and depletion, or recruitment and efflux, and are often evaluated by the expression changes of membrane proteins. CD4 is majorly expressed by helper cells, as well as by monocytes, macrophages, and DCs, and is used here to monitor helper cell alterations. CD4 expression does not change throughout the first 14 h after re-challenge, when compared to β-actin expression (Figure 17). TCR expression is a more T-specific marker but is not restricted to helper cells. TCR expression shows a similar expression down-regulation after 4 h that does not normalize in the following 10 h (Figure 17). Therefore, $CD4^+$ populations in the lung seem to be homeostatic even after allergen exposure. We have correlated the 4 h time point to increased DC antigen-presentation activity and would expect therefore lymphocyte activation and/or proliferation.

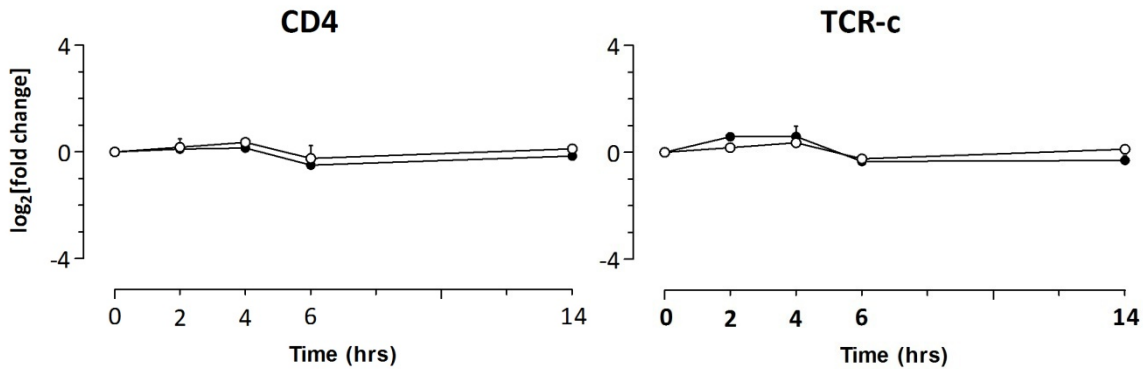


Figure 17. Expression of T helper cell membrane receptors CD4 and TCR were monitored in sensitized mice and show similar transcript expression levels and global regulation as β-actin baseline expression after re-challenge.

CD25-mediated lymphocyte proliferation signal is increased after allergen exposure and peaks at 4 to 6 hours after challenge.

The strong signal for cell proliferation of activated lymphocytes is mediated by the release of IL-2. Interestingly, the signal is transmitted from the activated cells themselves, that provides them with a stable enough proliferation signaling loop to make sure enough effector cells are accumulated to induce an adaptive response. T lymphocyte activation takes place after antigen-presentation, and up-regulates binding efficiency of the IL-2 receptor via CD25. This process is essential for every immune response elicitation. Significant CD25 up-regulation, when compared to β-actin expression (Figure 18), indicates lymphocyte proliferation after antigen-presentation, in general, and specifies the activation and proliferation dominant phase of our time course between 4 to 6 h after re-challenge and later.

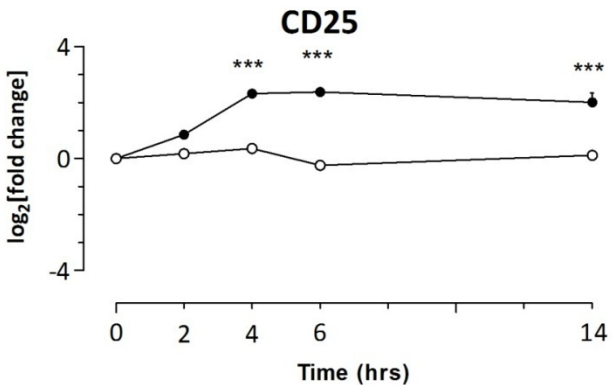


Figure 18. Expression of lymphocyte proliferation-promoting factor IL-2Rα is immediately up-regulated after allergen challenge. Expression of IL-2Rα associated to lymphocyte proliferation was evaluated and monitored during the early course of an asthmatic relapse. The curve seems to peak at 4 to 6 h and stays at high levels around 14 h after challenge.

Expression of major regulator of adaptive immunity GATA3 is decreased immediately after allergen challenge.

The event, when T helper cells differentiate into distinct types of subsets, reflects the moment of specific response type induction. Depending on diverse factors, $CD4^+$ naïve helper cells polarize into effector cells under the control of specific expression regulation mechanisms that are distinct in each subset of helper cells. Since GATA3 and Tbet are considered as major differentiation factor counterparts, we examined their expression, after allergen exposure to sensitized mice. This will help us to monitor which of the two major adaptive immunity subtypes is actively promoted after allergen challenge.

Regulation is an important part of every immune response and is often promoted shortly after elicitation of immunologic reactions. Regulatory cell effectiveness can be responsible for disease remission and might prevent adaptive immune response elicitation in auto-regulatory processes for which it is responsible at the first place. Its implication in IM is so crucial because regulatory mechanisms prevent accumulation of lymphocyte effector cells to excessive levels, and, if occurring early enough, might prevent the effector phase completely. If no effector cells are amplified, no memory cells are likely to develop.

Our results indicate no significant regulation change of FoxP3 after re-challenge, when compared to β-actin expression (Figure 19B). Interestingly, expression of GATA3 is kept at low levels, after an initial significant drop around 2 and 4 h after re-challenge, when compared toβ-actin expression (Figure 19A). Tbet expression, on the other side, is not significantly changed throughout the first 14 h after re-challenge, when compared to β-actin expression (Figure 19B).

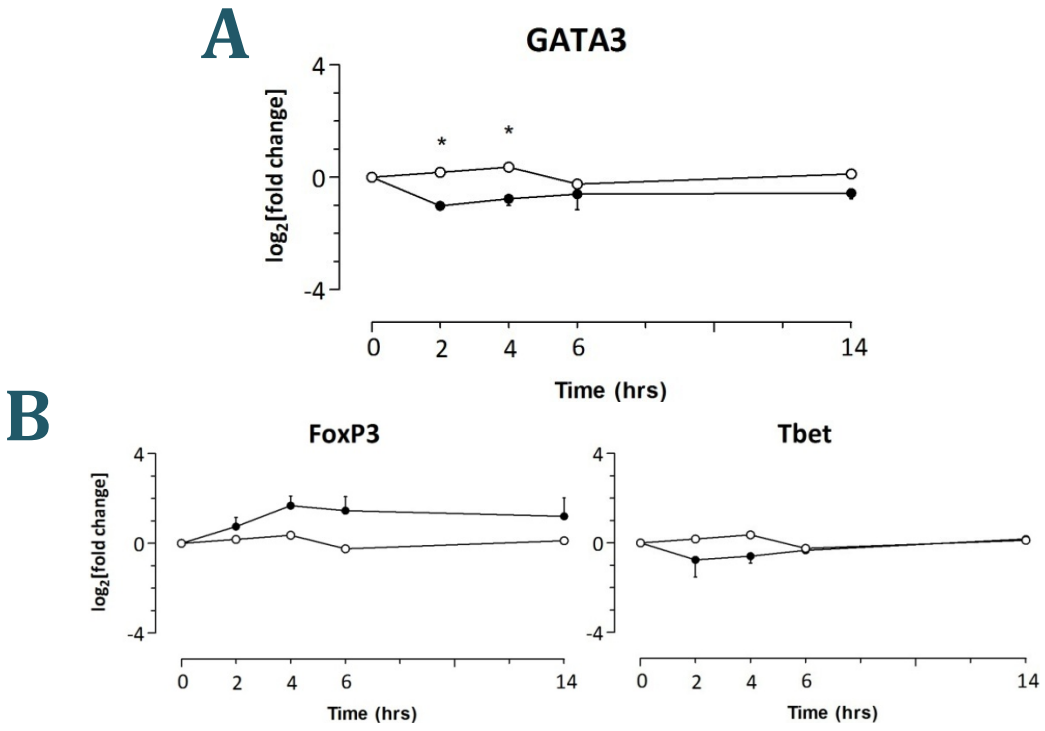


Figure 19. Transcription factors GATA3, FoxP3 and Tbet are monitored after allergen challenge. While (A) GATA3 expression is significantly down-regulated at 2 and 4 h after challenge and normalizes at the 6 h time point, expression of (B) Tbet shows no significant regulation trend. FoxP3 (B) on the other hand, shows a slight but statistically insignificant up-regulation trend after challenge.

Expression of Th2 related membrane receptors are down-regulated after re-challenge.

One of the most influential events during an immune response elicitation is the recruitment of effector cells to the site of inflammation. All upcoming processes are dependent on the cells that are present in the inflammation site in the following response progression. Since gene expression of Th2 cell differentiation regulators is decreased after re-challenge, genes of membrane receptors that are involved in the specific recruitment of Th2 cells were examined and show clear significant down-regulation, when compared to β-actin expression. CRTh2 expression reaches a stable minimum at 6 h after re-challenge. IL-25R expression starts normalizing, after significantly peaking downwards 6 h after re-challenge, when compared to β-actin expression (Figure 20A).

ST2 has been associated with lung and IgE-mediated inflammation. It has a crucial function in eosinophils. This receptor is mentioned here because it was supposed by other studies to be expressed by Th2 memory cells and to have major implications in asthma exacerbation. ST2 shows an up-regulation trend at 4 h after re-challenge (Figure 20B) that is not statistically significant, when compared to β-actin expression.

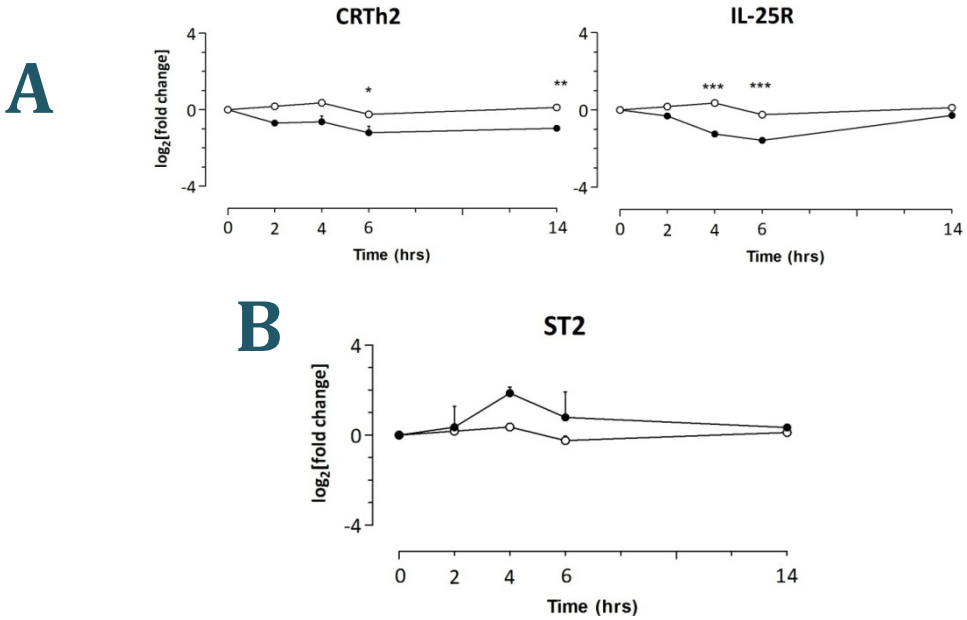


Figure 20. Membrane receptors of pro-inflammatory factors Prostaglandin D2, IL-25 and IL-33 associated with type 2 immune responses were evaluated. Expression of all these membrane proteins is assumed to be specific for Th2 cells. (A) CRTh2 and IL-25Rshowed increasing down-regulation. CRTh2 was down-regulated from the 6 h time point on. IL-25R expression showed a minimum at 4 and 6 h after challenge. Even though showing a slight up-regulation trend especially at the 2 and 4 h time point, ST2 showed no significant expression change after challenge.

After allergen challenge, CKRs are down- or up-regulated in distinctive reproducible expression patterns.

As antigen enters the body, respondent immune cells experience drastic changes in their physiology and molecular biology. One of these alterations concerns the navigation system of immune cells controlling their migration. Therefore, we examined gene activity of all known CKR genes after re-challenge of asthmatic mice. To distinguish between probable physiologically relevant and insignificant regulation trends we tested statistical differences of CKR and β-actin expression with two-way ANOVA.

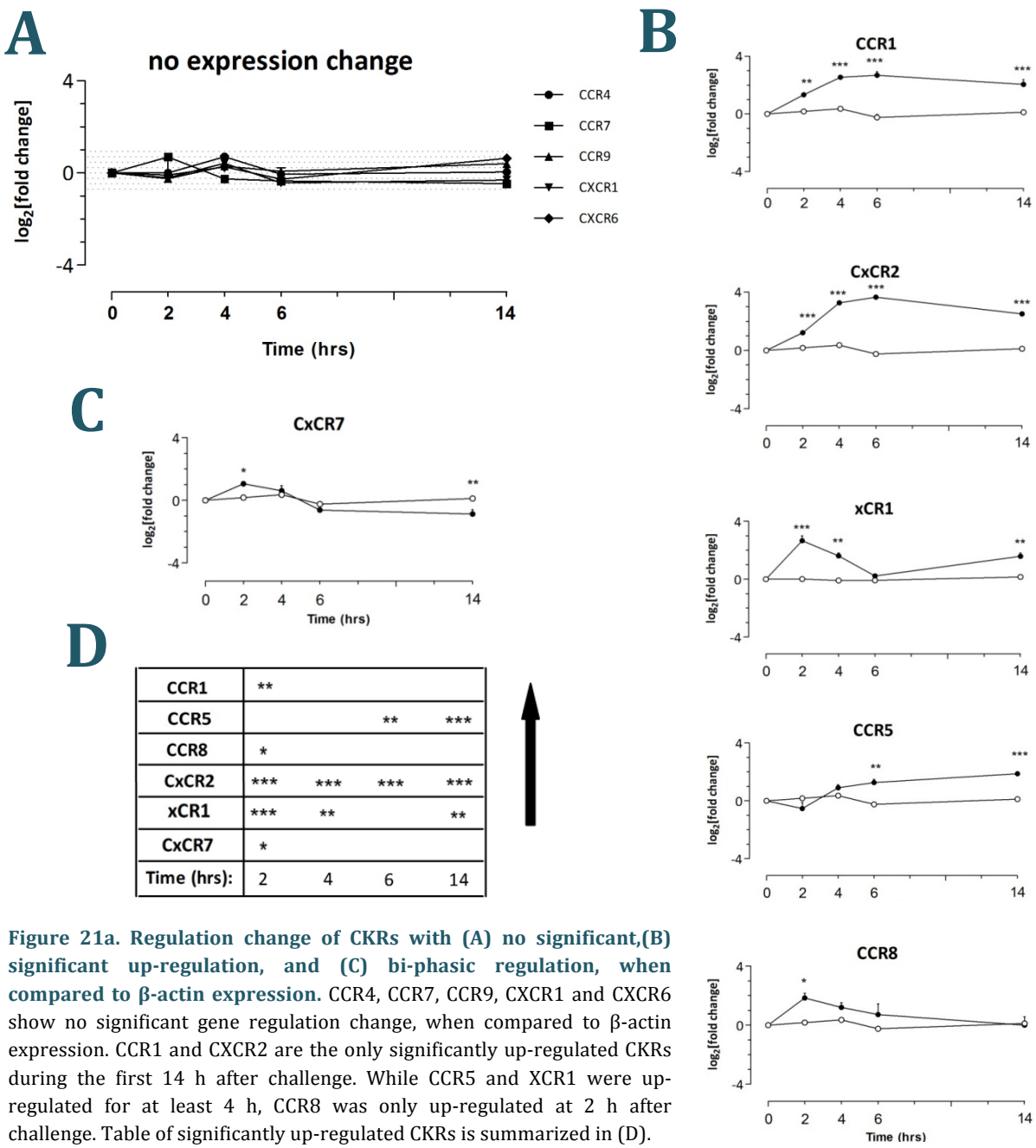
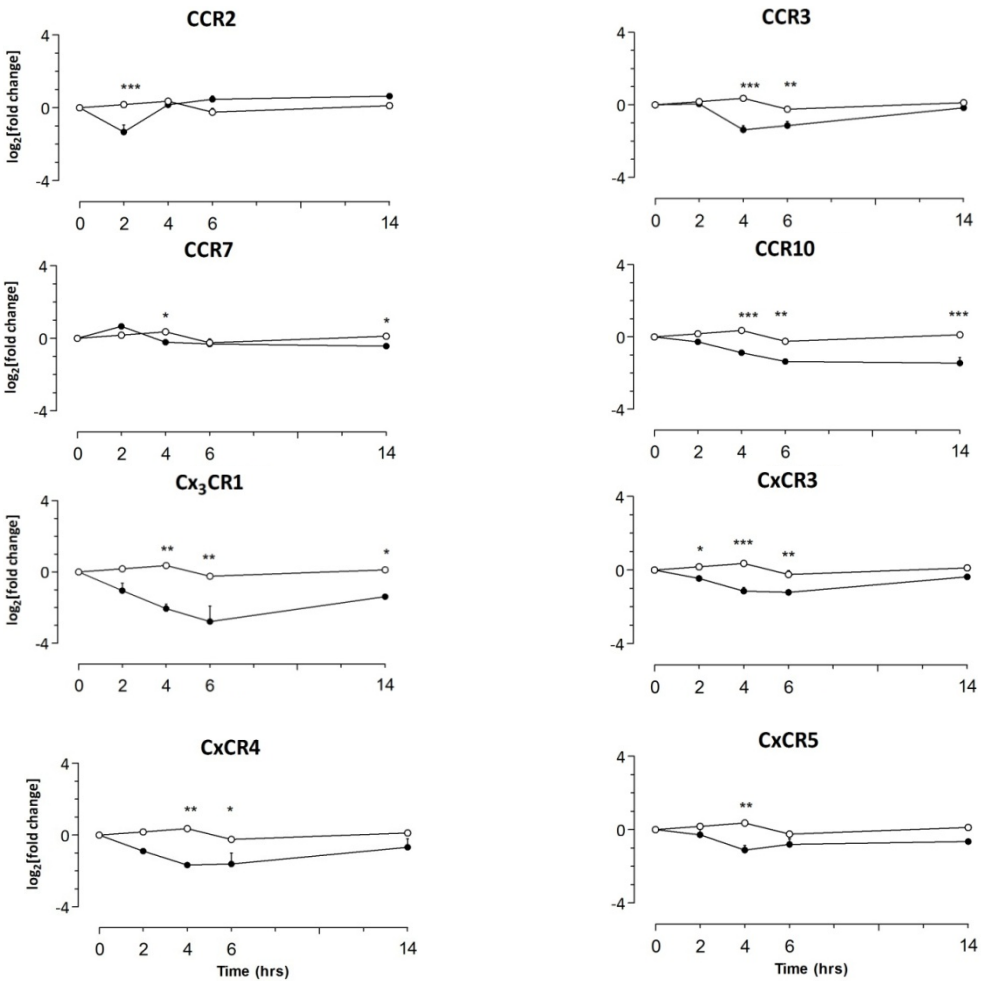


Figure 21a. Regulation change of CKRs with (A) no significant,(B) significant up-regulation, and (C) bi-phasic regulation, when compared to β-actin expression. CCR4, CCR7, CCR9, CXCR1 and CXCR6 show no significant gene regulation change, when compared to β-actin expression. CCR1 and CXCR2 are the only significantly up-regulated CKRs during the first 14 h after challenge. While CCR5 and XCR1 were up-regulated for at least 4 h, CCR8 was only up-regulated at 2 h after challenge. Table of significantly up-regulated CKRs is summarized in (D).

E



F

CCR2	***			
CCR3		***	**	
CCR7		*		*
CCR10		***	**	***
Time (hrs):	2	4	6	14

CxCR3	*	***	**	
CxCR4		**	*	
CxCR5		**		
Cx_3CR1		**	**	*
CxCR7				**
Time (hrs):	2	4	6	14

Figure 21b. Regulation change of CKRs with (E) significant down-regulation, when compared to β-actin expression. CCR10 and CX_3CR1 are the only significantly down-regulated CKRs during the whole time course after 4 h after challenge, while CCR3, CCR2, CCR7, CXCR3, CXCR4 and CXCR5 are at some point significantly down-regulated, but normalize within the investigated time course. CXCR7 is the only CKR with a bi-phasic gene regulation. Table of significantly down-regulated CKRs is summarized in (F).

No expression change. The group of genes which does not show at least 1-fold expression changes between neither of the time points is composed of CCR4, CCR7, CCR9, CXCR1 and CXCR6 (Figure 21aA).

Up-regulation. Receptors for typical inflammatory chemokines that were also related to T cell chemotaxis in other studies, XCR1, CCR8 and CXCR7 showed all a significant up-regulation of at least 1-fold intensity immediately after allergen exposure. All receptors experience a swift down-regulation trend from the 2 h time point onwards, except for XCR1 showing another up-regulation after 6 h after re-challenge.

From the five immediately up-regulated CKR genes two were CCR1 and CXCR2. In contrast to the other immediately up-regulated group, these CKR genes were constantly up-regulated throughout the first hours after re-challenge. CXCR2 is even showing the biggest up-regulation of all examined CKRs.

Expression of CCR5 stands out through is delayed regulation change after the 2 h time point onwards. After experiencing an immediate expression drop, CCR5 was noticeably up-regulated after 2 h after re-challenge. This up-regulation was maintained for hours and did not diminish during the examined period of asthma relapse (Figure 21aB).

Down-regulation. The last group of the remaining CKRs was divided into two groups according to the time point of their expression minimum. The first group is comprised of CCR3, CCR6, CXCR3, CXCR4 and CXCR5showing an early expression minimum around 4 or 6 h. These CKRs genes experience an equal significant down-regulation after re-challenge but all their expression normalizes at equal rates with proceeding time to the levels before allergen exposure.

The second group of down-regulated CKRs displayed an expression minimum from 6 h after re-challenge onwards. During the first period after allergen re-challenge CCR10, and CX_3CR1 were down-regulated for at least 10 h (Figure 21aE).

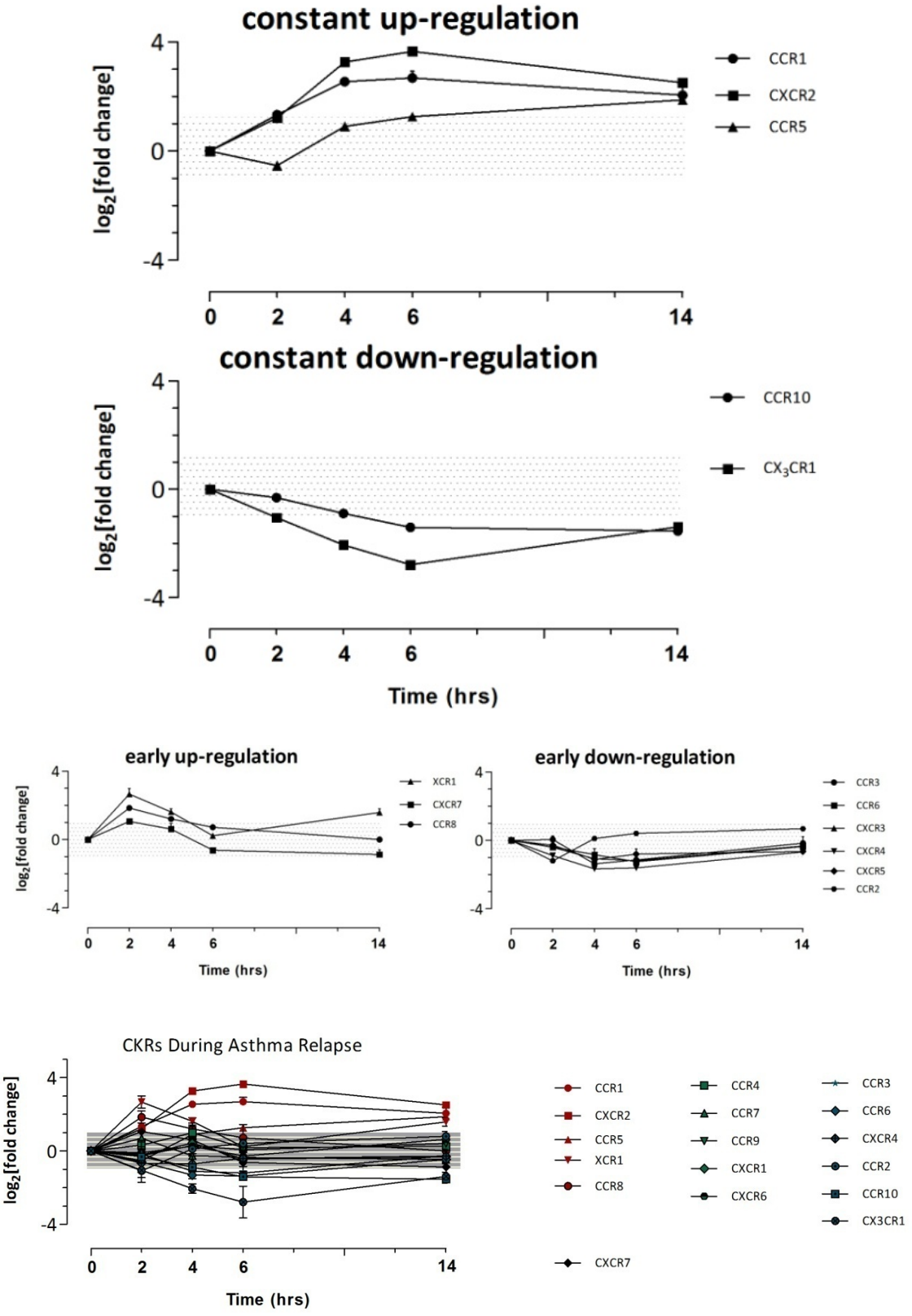


Figure 22. Patterns result in the smoothest way to understanding. Time dependent expression of CKRs might be major indicators for the course of an immune response. Understanding the mechanisms of immune cell activation, as asthmatic response is starting to elapse, might contribute to the understanding of the immune response itself.

DISCUSSION

Pulmonary marker gene expression in asthmatic mice

Residence of allergen-responsive memory T cells in peripheral allergen-exposed tissues makes sensitized individuals susceptible for uncomfortable chronic episodes of acute allergic responses, constant exposure-linked unease, and eventually severe physical impairments in the long run of routine contact with allergen. Immunologists estimate that asthma pathology starts to manifest, when differentiated bone marrow-derived cells of the adaptive immune system that populate the lymphatic system create an integrated microenvironment with structural cells and resident leukocytes inside lungs of asthmatic individuals. Even though these peripheral memory cell-containing infiltrates were shown to exist, not only morphological and cellular aberration is missing in lungs of mice with elevated levels of allergen-specific antibodies, also several patterns of global gene expression is surprisingly similarly regulated in healthy unaffected, and quiescent asthmatic mice alike. Conceivably, sensitivity for aeroallergen of the lung demands to be sketched at the level of other intrinsic perceptions about the tissue, i.e. type-restricted gene or cell membrane protein expression, to name only two.

Therefore, we evaluated global pulmonary gene expression for the difference between naïve and recovered mice in several immunity-related genes that are known to be active in differentiated lymphocytes. In lung samples from both naïve and recovered mice the expression of IL-4 and IL-13 could not be detected. Since these two cytokines are major mediators released by Th2 cells and dominate during acute allergic states, we confirmed our initial hypothesis that memory Th2 cells in recovered mice are in quiescent state. Interestingly, expression of common Th2 specific genes, like GATA3, CD4, ST2, CRTh2, IL-25R and IL-5, showed no significant difference between the groups. All of these genes were expressed at a high level compared to the housekeeping gene expression of HPRT. According to our estimations IL-5 showed the lowest expression that is approximately 1000 times less than HPRT. As we evaluate gene expression in the whole lung, it is possible that other cell types mask specific expression alterations of these genes by Th2 cells

through their expression. For instance, only Th2 cells express CRTh2 amongst lymphocytes. Gene expression, however, has also been shown in keratinocytes [151] and liver myofibroblasts [152]. Thus, we assume that distinct gene expression of homeostatic memory cell populations in asthmatic lungs can only be distinctly distinguished, if its expression is specific or strong enough in the front of a physiologic background gene expression. Expression of anti-OVA peptide TCR variable region α13 reflected therefore a desired candidate as specific cell marker, since its expression is restricted to T cells and was shown to be approximately 300-fold lower than expression of other investigated genes. Consequently, recovered mice showed increased expression of TCRα13$^+$antigen-specific receptor variants that could be statistically shown as highly significant in this group, especially when sample size was increased. We assume therefore that resident TCRα13$^+$cells could be good representatives for peripheral OVA-specific memory Th2 cells. Future work could investigate expression of this and other markers on individual cells in the lung, using methods such as flow cytometry or immune fluorescence. These methods would specifically allow evaluation of the expression intensity of membrane proteins on CD4$^+$ cells, and might therefore elucidate the relation between specific gene expression and allergen-specificity of lymphocytes. Additionally, proteins that could characterize the targeted memory cells specifically further are factors that enhance responsiveness to allergens (i.e. vitamin D receptor [61]), enable physical attachment to epithelium or myelocytes (i.e. adhesion molecules, integrins [153]), or regulate homeostatic proliferation (i.e. nuclear factors), to name a few. Alternatively, expression of TCRβ8.1/2$^+$ cells that form functional OVA-specific TCRs with TCRα13 sub-chains could be evaluated, to monitor immunity against OVA [154].

Differential CKR gene expression in lungs of asthmatic mice

If identity of a cell population with a distinct function were self-evident with our experimental techniques today, the quest for a cell marker would not be necessary at all. As a consequence of this shortcoming, cell-specific expression cannot be unobjectionably determined, unless the single cell is investigated alone in absolute unaltered native environment. The strength of this study is that our

approach includes the unaltered native environment of target cells into the measurement. Consequently, we were concerned that this would blur out cell-specific gene expression at the same time. On second glance however, we had observed that it is this similarity between naïve control mice and recovered mice that actually gave us the possibility for revealing *asthma-related gene expression alterations* of the test groups *on a global scale*, including neuronal, muscular and structural tissue contribution to the whole pulmonary system. Thus, the robust indications that OVA-specific T cells are situated in recovered lungs of asthmatic mice (through previous studies mentioned in the introduction, and our finding about the $\alpha 13^+$ TCR expression) led us to extend our focus on the expression of the whole array of CKR genes known so far, in order to compare asthma-related differences between the two groups to find statistically significant changes.

While CCR6 seemed to be significantly down-regulated by a 151% expression difference, CCR9 was the only expressed CKR gene that increased in the lungs of asthmatic mice. The increase averages around 118%. In former studies CCR9 expression, besides being associated to increased specific cell homing to gut mucosa [155], was shown to increase expression of selected integrin chain subtypes on $CD8^+$ lymphocytes, after being induced by all-trans retinoic acid via intracellular retinoic acid receptor in vitro [156] itself. The only known ligand for CCR9 is CCL25 [150]. We assume that the CCR9-CCL25 signaling axis might be implicated in asthma perpetuation, or even the retention of allergen-specific memory cells in the lungs of asthmatic mice. CCR9 was shown to be involved in a mechanism that provides selective modulation of lymphocyte and epithelial cell interaction [157]. Consequences of manipulating this signaling axis in asthmatics are difficult to be anticipated, since double positive thymocytes and $TCR\gamma\delta^+$ T cells were already shown to express CCR9. Furthermore, NKT cells are active modulators of asthma and their migration to the lungs of asthmatic individuals was shown to depend on the expression of CCR9 [130].

CCR6 has also been identified to possess a single ligand so far, named CCL20. This CKR has been associated with migration of Tregs [158], Th17 cells [123] and DC progenitors [159] and was interestingly shown to be down-regulated by DCs, when IL-4 and TNFα is present [160]. Our observation that the two monogamous chemokine

receptors are differentially expressed in asthmatic lungs (implicitly in response to treatment with OVA) puts their character and restricted ligand specificity in question for being the responsible denominator of the detected expression changes.

These findings need to be investigated further, to elucidate the responsible cell types that cause the altered gene expression in recovered mice. Co-expression with CCR6 or CCR9 with cell lineage-restricted markers through flow cytometry or immunofluorescence are necessary to identify which cell type is affected by the treatment with OVA. Functional test through blocking or depleting these receptors or their ligands might give further indication about their relevance to the asthma phenotype. Extraction of different lines of $CCR6^+$ and $CCR9^+$ cells can also reveal the role of these CKRs in the induction of AA, when they are transferred into healthy mice. As we evaluated the change in the whole pulmonary gene expression, masked asthma-related CKRs have also to be identified on the level of the single cell, i.e. by flow cytometry.

Th2 cytokine expression increases early after allergen re-exposure

Former studies revealed that maintenance of allergen-specific memory cells in the lungs of asthmatic mice lasts for a lifetime [6]. When these mice inhale aerosolized allergen, the altered microenvironment in their lungs causes elicitation of an allergen-specific response that lasts for days. Intercellular communication that induces fast and global changes in cell activation is generally mediated by secretion of cytokines and chemokines. IL-4 expression of each experimental subject was therefore monitored and seemed to be predominantly inflammatory and immensely up-regulated in unexceptionally every allergen-exposed asthmatic mouse. As control HPRT, β-actin and GAPDH expression was confirmed not to be significantly different between expression in mice before and after re-challenge. Relative β-actin expression was consequently used to evaluate statistical changes of target gene to baseline expression.

We could show that cytokines dominating the early hours after re-challenge is a set of interleukins characteristically secreted by type 2 helper cells. In comparison to IFNγ – major Th1 type cytokine significantly up-regulated at 4 and 6

h after re-challenge – asthma-promoting cytokines IL-4, IL-5 and IL-13 are immensely and constantly up-regulated immediately after allergen exposure. IL-4 experiences the most profound up-regulation trend that exceeded a 14,000-fold increase in all cases of re-challenged mice at the 2 h time point. Up-regulation of IFNγ might be a by-stander effect of pro-inflammatory cytokine release on Th1 cells, but might also enhance the Th2 type response by inducing up-regulation of MHCII and antigen-presentation to Th2 cells.

The expression patterns of T helper cell cytokines confirmed that asthma relapse might ubiquitously be uniform in elicitation and similarly regulated, both in time and intensity. According to literature, typical late phase reactions mediated by activity of resident Th2 cells in the lower airways start developing 2 h after allergen exposure. In this period of response elicitation, lymphocyte activation by antigen-presentation and effector cell proliferation starts taking place (below more). Therefore, respondent naïve helper cells cannot be held responsible for the immediate rise in Th2 cytokine expression. The responsible mechanism for this fast up-regulation of cytokine gene expression must be related to IM. The up-regulation is happening instantly after allergen exposure, and before an expanding lymphocyte population could have caused it. The specificity of this response was confirmed via comparison to BSA-challenged asthmatic mice showing completely different cytokine expression patterns. This suggests one more time that IM is implicated in responses of sensitized animals to allergen.

Interpretation of isolated events in a complex process like an immune response can be misleading unless the context is not given. This can be tackled in our approach by considering factors of tissue response and activation of the innate immune system, for instance. Presence of inflammatory cytokines is assumed to affect structural cells and cells of the innate immune system. Expression of TSLP and IL-33 is associated with promotion of Th2 type responses partially through direct action on Th2 cells [161]. We could detect a significant up-regulation for both genes peaking between 4 and 6 h after re-challenge. We suspect that this phase represents the second wave of promotion towards a Th2 type response and occurs intensively during this period of time, featured by stimulated antigen-presentation and unfolding of asthma phenotype. Presumably, also integration of diverse signals from

antigen-presentation *and* global response amplification takes place beginning from 4 to 6 h onwards after re-challenge. During excision of the lungs we could observe that pro-inflammatory chemokines eotaxin-1 and eotaxin-2 were also evaluated showing a steady increase in gene expression that might eventually lead to increased eosinophil recruitment.

Tracking helper T cells during acute asthma relapse

Even though a multiple number of signals from diverse sources are needed for a complete asthmatic response elicitation, the driving force behind allergic responses is widely considered to be T cell effectiveness. To get an impression of the total T cell population and more specifically of helper cells, we evaluated the expression of the constant region of TCRs and CD4 after re-challenge. We could not point out any significant gene expression change of these proteins of the antigen-receptor-associated membrane complex in the lungs of asthmatic mice after allergen re-exposure. While absolute T cell infiltration could not be confirmed, we assume that significant up-regulation of CD25 gives a clear indication for mounting lymphocyte proliferation starting around 4 h after re-challenge. A possible course of asthma relapse might therefore be lined out by early Th2 cytokine production, following lymphocyte proliferation and finally eosinophil recruitment. These three events require further specific assays to address elucidation of their induction pathway, elicitation time point and extent of impact.

The exact activation mechanism of memory cells is still elusive, even though there are several indications that this event is happening relatively fast after antigen re-exposure. The conventional activation of naïve T cells includes antigen uptake and presentation, as well as, following cell stimulation and proliferation. Effector phase of T cells requires distinct differentiation of activated cells into helper cell subtypes. Precise regulation of differentiation-linked gene expression patterns underlies the presence of diverse nuclear factors that are orchestrated by master transcription factors. We evaluated typical master transcription factors of Th1 cells, Th2 cells and Tregs after re-challenge, to monitor those populations in the lungs after allergen re-challenge. Elevated pulmonary expression of any of these genes

could signify increased influx of cells that are expressing them. It could also signify ongoing differentiation or proliferation. With regards to GATA3 expression, we assume that its significant down-regulation during the first 6 h after re-challenge might either be actively induced or obey an indirect causality. Both cases could be explained by fast proliferation of GATA3 expressing cells that actively decrease GATA3 expression, or dilute the total expression product during clonal expansion, or both.

FoxP3 was the only transcription regulator with an increased expression trend. This trend however had no statistical significance. The cause for this slight increase might or might not be augmented cell infiltration of activated Tregs into the exposed lung. Another speculation is that this weak trend could be an effect of increased auto-regulatory processes taking place to control the already mounting secondary response. If we assume that this elevated auto-regulation is a mere response to the presence of an antigen, we could speculate, that unspecifically elevated effectiveness of Tregs might be an additional mechanism to decrease the possibility for elicitation of an adaptive immune response. Adaptive responses always have the possibility to install IM in the organism with far-reaching consequences. As discussed in the entry chapter, IM can mean a complete physiological change for an organism and must therefore be tightly regulated. *After* IM has already installed in peripheral tissue, presence of antigen will always induce strong pro-inflammatory dynamics that precede this auto-regulatory mechanism, and clear the path for an adapted secondary immune response.

On the one hand, we could observe that Th2 cytokine expression is immensely up-regulated upon allergen exposure. Activated peripheral allergen-specific Th2 memory cells in the lung tissue of asthmatic mice might induce this up-regulation. On the other hand, we have indications for lymphocyte proliferation in the early hours after re-challenge, while lymphocyte differentiation shows no signs of being in progress yet and GATA3 expression seems to be significantly decreased. Precise behavior of peripheral allergen-specific Th2 memory cell populations in the lung tissue still remains elusive. To track the response of Th2 memory cells, we evaluated cell subtype-associated membrane proteins ST2, IL-25R and CRTh2. Assessment of IL-25R and CRTh2 showed significant down-regulation of gene

expression after re-challenge. These trends might be connected to either proliferative side effects or the exit of activated Th2 lymphocytes from the exposed lung, for instance. ST2 expression shows an opposite expression trend and has yet no statistical significance. These paradox results for Th2 membrane protein expression could possibly have a physiological explanation. Expression of ST2 was shown to be dependent on proliferation in fibroblasts [162]. If these two results are associated with each other then it would be comprehensible that ST2 behaves CD25-like after re-challenge, while ongoing processes in response to allergen exposure demand expression down-regulation for specific genes without relevance to expansion, like CRTh2 and IL-25R. Interestingly, gene expression patterns of ST2 and IL-33 are similar and peak around the same time point after allergen re-challenge. ST2 gene expression might therefore be correlated to presence of IL-33 or positively enhanced through a regulatory feedback mechanism.

Pulmonary CKR gene expression during acute asthma relapse

Determining important gene expression patterns in different phases of AA in mice is apparently crucial for understanding the consequences for asthmatics in contact with aeroallergen. Furthermore, it will bring us closer to elucidation of the role of CKRs in disease perpetuation and relapse. Thereby, we evaluated all known CKRs after re-challenge and detected five genes that were either definitely down- or up-regulated: CCR10 and CX_3CR1 were down-regulated and CCR1, CXCR2 and CCR5 were up-regulated.

CCR10 is expressed by lymphocytes with skin-tropism and CX_3CR1 was shown to be essential for DC-epithelial cell interaction[163] and is also expressed by $CD16^+$ natural killer cells and $CD8^+$ CTLs. Thus, recruitment or activation of these cells is not suggested by our results. Interestingly, smooth muscle cells, epithelial cells, and neutrophils presumably express the two genes with the most up-regulated gene expression. CXCR2 up-regulation might be a good indicator for neutrophil invasion directly after allergen exposure, and was clearly detectable in comparison to eosinophil expressed CCR3. Involvement of structural cells in the development of the asthma phenotype is indicated by the immense up-regulation of CCR1 whose

ligands are mediators of asthma pathology [112]. Blocking of CCR5 has been shown to promote the asthma phenotype [121]. CCR5 is supposedly expressed with CXCR3 on Th1 cells. Noticeably, CXCR3 shows a slight down-regulation trend during the first period after re-challenge. Other up-regulated genes are xCR1 and CCR8. Both are expressed by lymphocytes and might become interesting in functional tests. While CCR8 is associated to effector Th2 cells, XCR1 was shown to have regulatory functions because its ligand is expressed by Tregs [110]. One hypothesis is that XCR1 expression is immediately up-regulated and drops then at 6 h after re-challenge because its expression was shown to be inversely regulated to Treg effectiveness.

CXCR7 is interesting because it is the only CKR that shows a bi-phasic expression. Since it is not expressed by lymphocytes, its expression might be an indicator for the transition between different phases after allergen exposure by yet unidentified cell types, presumable endothelial cells [164].

Interpretation about differential expression of the down-regulated CKRs is more vague with our current knowledge. Down-regulation of CCR7 might indicate a lowered declination for homing to the lymph nodes or exit of $CCR7^{+}$ cells from the exposed lung tissue. CCR3 down-regulation might be a side effect of neutrophil recruitment during the early phases of allergen exposure. Interestingly, CCR2 showed immediately an immense down-regulation in the range of approximately 230%. Paradoxically, CCR2 expression has been associated with the recruitment of pulmonary DC precursors during allergic airway inflammation [165]. If connected to DCs, expression drop could be associated to DC exit from the lung after activation. This immediate down-regulation, however, is followed by an estimated expression increase by 390% from the 2 h time point onwards after exposure to allergen. Amongst others, this delayed but intense up-regulation might therefore be connected to altered activation of DCs or T cells located in the re-challenged asthmatic lung.

Conclusions

1. Comparing gene expression between the lungs of naïve mice and mice recovered from allergic asthma we determined that:

- Vα13^{+} TCR variant expression was a reliable marker for the presence of OVA-specific memory T cells in the lung tissue of recovered mice
- Expression of CCR9 was shown to be significantly increased and expression of CCR6 significantly down-regulated in the lung tissue of recovered mice compared to naïve healthy controls.
- Expression of all other CKRs was not differently expressed.

These results imply that CCR9 might be involved in the maintenace of memory Th2 cells in the lungs of recovered mice, which should be further validated.

2. Comparing gene expression between the lungs of recovered mice and mice that were re-challenged with allergen for 2, 4, 6 and 14 h we determined that:

- Expression levels of all Th2-specific cytokines, IL-4, IL-5 and IL-13, were immensely up-regulated after allergen re-challenge
- IFNγ showed a significant up-regulation between 4 and 6 h after allergen exposure of asthmatic mice
- Several genes of secreted asthma promoting factors (TSLP, IL-33 and eotaxins-1 and -2) showed clear up-regulation, while specific genes of Th2 cells were either not affected (ST2) or down-regulated (CRTh2 and IL-25R).
- Expression of CCR4, CCR7, CCR9, CXCR1 and CXCR6 did not change after re-challenge at all
- After allergen re-challenge pulmonary gene regulation patterns of CCR1 and CXCR2 showed clear up-regulation from 2 h and patterns of CCR10 and CX_3CR1 showed clear down-regulation from 4 h after contact with allergen.

These results imply that an immense Th2-mediated immune reaction is initiated in the lungs within 2 h after allergen rechallenge. Furthermore, substantial changes in Th2 marker and CKR gene expression provide insight in early immune response after allergen rechallenge and warrant new experiments in which expression of CKRs would specifically be determined on CD4+ T cells.

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ZUSAMMENFASSUNG

DAS immunologische Gedächtnis befähigt das Immunsystem eines Organismus schneller, effektiver und in erhöhtem Ausmaß auf ein Antigen zu reagieren, gegen welches es bereits Abwehrkräfte entwickeln musste. Wenn es zu einer Immunantwort kommt, können bereits auf die erste Aufnahme eines Antigens hin Gedächtnis-Lymphozyten im organischen Gewebe heranreifen, die diese Wiedererkennungsleistung vollbringen. Weithin als „memory“ Lymphozyten bekannt, können diese B-Zellen und T-Helferzellen einen wichtigen Bestandteil eines lebenslangen Schutzes gegen Pathogene bilden und daher lebenserhaltend bei wiederkehrenden Infektionen wirken. Andererseits können Gedächtnis-Zellen auch eine pathologische Rolle spielen. Das Immunsystem von atopischen Patienten besitzt die Tendenz auf den Kontakt mit ungefährlichen Stoffen aus der Umwelt, wie Pollen und Staub, Abwehrreaktionen zu zeigen. Diese Stoffe werden als Allergene bezeichnet, die im Falle von atopischen Individuen bei ausgedehntem Kontakt zur Anhäufung von $CD4^+$ T-Helferzellen – also Th2-Zellen – führen, die spezifisch auf das Allergen reagieren und die Entwicklung von Allergien verursachen. Die von allergenspezifischen Th2-Zellen ausgelöste Reaktionskaskade beinhaltet u.a. einen Anstieg des IgE Antikörpertiters im Blut, sowie Eosinophilie im betroffenen Gewebe und umliegenden Interstitium.

Welche Auswirkungen der Kontakt mit Allergenen haben kann, ist sehr uneinheitlich und hängt vom Organ ab, das dem Allergen ausgesetzt wird, sowie von der Natur des allergenen Stoffes selbst. Patienten mit allergischem Asthma – abgekürzt AA – leiden unter wiederholten Phasen von Kurzatmigkeit, Husten und Keuchen, aufgrund schwerer chronischer Entzündungen der Atemwege, Überproduktion von Lungensekret, bronchialer Überempfindlichkeit und reversiblen Atemwegsverstopfungen. Die Rolle, die „memory” T-Helferzellen bei allergischen Reaktionen spielen, wurde durch die Beobachtung ersichtlich, dass die gezielte Vernichtung dieser Zellen die Entstehung der Anzeichen von Allergien verhindert, während ihre Übertragung in einen gesunden Wirt die Krankheit passiv einzuleiten vermag.

Verschiedene AA-Maus Modelle stehen zur Verfügung. Akute Entzündungsreaktionen der Lunge können durch das Aussetzen systemisch sensibilisierter Mäuse mit Antigenaerosol hervorgerufen werden. Diese Reaktionen ähneln den Symptomen von AA beim Menschen. Nach dem Abklingen der akuten Phase von AA, weisen die – im Folgendem – als „erholt“ bezeichneten Mäuse verglichen mit naiven Mäusen Zellinfiltrate in der Lunge vor. Des Weiteren sind erhöhte Werte der IgG1 und IgE Antikörpertiter des Blutserums bei Asthma-Mäusen zu verzeichnen. Bemerkenswerterweise gilt, dass diese Zellinfiltrate in den Lungen erholter Mäuse antigenspezifische Gedächtnis-Zellen enthalten. Frühere Arbeiten zeigten, dass diese Gedächtnis-Zellen das gesamte Leben der erholten Mäuse hindurch im Lungengewebe verbleiben und bei erneutem Aussetzen mit Antigenaerosol einen Rückfall mit Anzeichen allergischer Anfälle verursachen.

ZIELE Die generellen Eigenschaften einer immunologischen Reaktion werden bestimmt durch den Typus, der Zahl und der Position der involvierten Zellen. Es bestehen unzählige Hinweise darauf, dass chemotaktische Zytokine – genannt Chemokine – eine der Hauptrollen in der Rekrutierung, Positionierung und Aufrechterhaltung von migrierenden Immunzellen spielen. Das Ziel dieser Arbeit ist es zu ermitteln, welchen Einfluss das Chemokin-System auf diese Funktionen bei antigen-spezifischen ‚memory’ Th2 Zellen im Lungengewebe in den unterschiedlichen Phasen von AA spielt. Weiterhin werden Expression einiger Gene ermittelt, die Hinweise auf Präsenz und Aktivierung von antigenspezifischen ‚memory’ Th2 Zellen in der Lunge geben, mit unter anderem Th2-spezifische Zytokine und T Zell Aktivierungsmarker. Zur Untersuchung der Aufrechterhaltung von antigenspezifischen

‚memory' Th2 Zellen durch Chemokinrezeptoren wurde die Genexpression der Lunge von Mäusen nach der Erholung von einer akuten asthmatischen Reaktion und von gesunden unbehandelten Mäusen verglichen. Zur Bestimmung der Rolle erwähnter Gene während eines Rückfalls wurden Mäuse mit Allergenerfahrung vor und nach erneutem Aussetzen mit dem Allergen verglichen.

METHODEN Wir ermittelten Unterschiede in der Genexpressionen für Chemokinrezeptoren und ausgewählten Markern zwischen dem pulmonalen Transkriptom von gesunden (naiven) Mäusen, erholten Mäuse nach einer akuten asthmatischen Reaktion (recovered), sowie von Mäusen mit Allergenerfahrung nach dem erneutem Aussetzen des Antigens (re-challenged) mittels Echtzeit-PCR.

ERGEBNISSE Die variable TCR region α13 und die monogamen Rezeptoren CCR6 und CCR9 zeigten deutliche Genexpressionsunterschiede zwischen gesunden und erholten asthmatischen Mäusen. Während TCR α13 und CCR9 eine erhöhtes Genexpressionsniveau aufzeigten, war die Expression von CCR6 signifikant in asthmatischen Mäusen vermindert. Die Expressionsstufe aller typischen Th2 Zytokine Interleukin-4, -5 und -13 wurden nach Aussetzen mit dem Allergen immens gesteigert, während eine erhöhte Expression von Interferon γ nur innerhalb von 4 und 6 Stunden nach Re-exposition zu verzeichnen war. Die Gene einiger sekretierter Faktoren (TSLP, Interleukin-33 und Eotaxin-1 und -2), die asthmatische Anfälle fördern, wurden deutlich hochreguliert. Zeitgleich blieben spezifische Membranproteine von Th2 Zellen entweder unverändert (ST2) oder zeigten sogar verminderte Expression (CRTh2, Interleukin-25 Rezeptor). Die ermittelte Chemokinrezeptorexpression bis zu 14 Stunden nach Inhalation des Allergens wies auf das Bestehen diverser Regulationsmuster hin. CCR1 und CXCR2 wurden nach 2 Stunden eindeutig hochreguliert. CCR10 und CX_3CR1 wurden ab der 4. Stunde nach Aufnahme des Allergens herunterreguliert. Wir beobachteten ebenfalls eine Gruppe von Genen, die nicht auf die Allergenexposition reagierten (CCR4, CCR7, CCR9, CXCR1 and CXCR6).

SCHLUSSFOLGERUNGEN In der Gesamtheit der untersuchten RNA Expression der Lungen asthmatischer Mäuse war die Genexpression der $vα13^+$ TCR Seitenkettenvariante ein zuverlässiger Marker für die Präsenz von allergenspezifischen ‚memory' Th2-Zellen. Unsere Ergebnisse deuten des weiteren darauf hin, dass CCR9 womöglich im Zurückbleiben dieser Gedächtnis-Zellen in asthmatischen Lungen mitspielt, was nach weiteren Untersuchungen zur genauen Ermittlung des Ausmaßes seiner Rolle verlangt. Auf die wiederholte Inhalation des Allergens hin wies die pulmonale Zytokingenexpression auf die Bildung einer starken Th2 zellvermittelten allergenspezifischen Immunantwort hin, bereits 2 Stunden nach der Exposition. Expressionsmuster diverser asthma- und lymphozytenbezogener Markergene trug zu weiteren Einsichten in die frühen Ereignisse nach einer Allergen Re-exposition bei. Pulmonale Genexpression zeigte für alle bekannten Chemokinrezeptoren nach Aufnahme des Allergens ein sich änderndes Muster. Diese Änderungen waren jedoch nur für CCR1, CXCR2, CCR5, CCR10 und CX_3CR1 von Dauer. Aufgrund der Wiederholungen von Expressionsverläufen vermuten wir, dass es eine begrenzte Anzahl bestimmter Regulationsmuster gibt, denen bestimmte Chemokinrezeptorgene folgen. Die Gruppe von Chemokinrezeptoren, die nicht auf die Aufnahme von Allergen Genexpressions-veränderungen aufzeigten (CCR4, CCR7, CCR9, CxCR1 and CxCR6), sind daher möglicherweise nicht für die Einleitung einer asthmatischen Reaktion notwendig. Wir erhoffen uns, dass aus den gewonnenen Einsichten unserer Untersuchungen die Herstellung spezifischer Pharmazeutika sowie Angriffsziele zur Behandlungen von rückkehrenden asthmatischen Erscheinungen auf den Kontakt mit Aeroallergen entstehen könnten.

CURRICULUM VITAE

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RESEARCH EXPERIENCE

Vienna Biocenter, Max F. Perutz Laboratories, Department Infection Biology, Leiter Dr. Thomas Decker.
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Medizinische Universität Wien, Universitätsklinik für Dermatologie, Labor für Experimentelle Allergologie, Leiterin Dr. Michelle Epstein.
RNA Extraktion aus formalin-fixierten paraffin-gebetteten Geweben und RNA Qualitätsbestimmung, Dezember 2008 – Jänner 2009

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Laborpraktikum Zusammensetzung der Untereinheiten des nicotinischen Acetylcholinrezeptors, Februar – Mai 2009

Seibersdorf Laboratories, Experimentelle Abteilung der Internationalen Atomenergiebehörde Wien, Pflanzenzuchtseinheit des Department of Nuclear Sciences and Applications, Agency's Laboratories.
Laborpraktikum Feldfruchtoptimierungen (crop improvement) an der Bananenstaude M. Acuminata, April – Juni 2009

Medizinische Universität Wien, Universitätsklinik für Dermatologie, Labor für Experimentelle Allergologie, Leiterin Dr. Michelle Epstein.
Diplomarbeit über Die Charakterisierung von Th2 Zellen und relative Chemokinrezeptor Expression während bestimmten Phasen allergischer Asthma, April 2011 – März 2011

PUBLIKATIONEN und PRÄSENTATIONEN

Berislav Bosnjak, Danilo Licastro, Mohammad N. Elagabani, Lamia El-Housseiny, Elia Stupka, Michelle M. Epstein. *Manuscript: Gene expression profiling of mice with allergic asthma. ÖGAI Jahrestagung, Verleihungen der Landsteiner-, Pirquet- und Dissertationspreise*

RESEARCH INTERESTS

Expression von Membranrezeptoren zur Entwicklung von zellkommunikationsbasierten Mechanismen und Entstehung von integrativen Fähigkeiten wie Gedächtnis und Erinnerung

Neuroscience, Immunologie, Zellbiologie

CHEMOKINE GLOSSARY

C-C-CHEMOKINES•

CCL1 (P500; SISe; TCA3; I-309; SCYA1)

This cytokine is secreted by activated T cells and displays chemotactic activity for monocytes but not for neutrophils. It binds to the chemokine receptor CCR8.

CCL2 (HC11; MCAF; MCP-1; SCYA2; GDCF-2; SMC-CF)

This cytokine displays chemotactic activity for monocytes and basophils but not for neutrophils or eosinophils. It has been implicated in the pathogenesis of diseases characterized by monocytic infiltrates, like psoriasis, rheumatoid arthritis and atherosclerosis. It binds to chemokine receptors CCR2 and CCR4.

CCL3 (MIP1A; SCYA3; G0S19-1; LD78ALPHA)

The encoded protein plays a role in inflammatory responses through binding to the receptors CCR1, CCR4 and CCR5. Polymorphisms at this locus may be associated with both resistance and susceptibility to infection by human immunodeficiency virus type 1.

CCL4 (ACT2; G-26; LAG1; MIP1B; SCYA2; SCYA4; MIP1B1; AT744.1)

CCL5 (SISd; SCYA5; RANTES; TCP228)

The cytokine encoded by this gene functions as a chemoattractant for blood monocytes, memory T helper cells and eosinophils. It causes the release of histamine from basophils and activates eosinophils. This cytokine is one of the major HIV-suppressive factors produced by $CD8^+$ cells. It functions as one of the natural ligands for the chemokine receptor CCR5 and it suppresses in vitro replication of the R5 strains of HIV-1, which use CCR5 as a coreceptor. Expression of CCR5 and CCL5 in synovial fibroblasts was shown to increase IL-6 production during osteoarthritis which stimulates osteoclast formation [166]. Correlation between levels of factors for rheumatoid arthritis in the serum of patients and CCR5 expression of DCs from peripheral blood brought the idea to use CCR5 expression on these cells in the monitoring of treatment efficacy [167].

CCL6 (c10; MRP-1; SCYA6)

CCL7 (FIC; MARC; NC28; MCP-3; SCYA6; SCYA7)

This gene encodes monocyte chemotactic protein 3, a secreted chemokine which attracts macrophages during inflammation and metastasis. The protein is an in vivo substrate of matrix metalloproteinase 2, an enzyme which degrades components of the extracellular matrix.

CCL8 (HC14; MCP-2; SCYA8; SCYA10)

Cytokines are a family of secreted proteins involved in immunoregulatory and inflammatory processes. The protein encoded by this gene is structurally related to the CXC subfamily of cytokines. Members of this subfamily are characterized by two cysteines separated by a single amino acid. This cytokine displays chemotactic activity for monocytes, lymphocytes, basophils and eosinophils. By recruiting leukocytes to sites of inflammation this cytokine may contribute to tumor-associated leukocyte infiltration and to the antiviral state against HIV infection.

• provided by RefSeq

CCL9 (CCF18; MRP-2; SCYA9; SCYA10)

CCL11 (SCYA11)

The cytokine encoded by this gene displays chemotactic activity for eosinophils, but not mononuclear cells or neutrophils. This eosinophil specific chemokine assumed to be involved in eosinophilic inflammatory diseases such as atopic dermatitis, allergic rhinitis, asthma and parasitic infections.

CCL12 (MCP-5; SCYA12)

CCL13 (NCC1; CKb10; MCP-4; NCC-1; SCYL1; SCYA13)

The cytokine encoded by this gene displays chemotactic activity for monocytes, lymphocytes, basophils and eosinophils, but not neutrophils. This chemokine plays a role in accumulation of leukocytes during inflammation. It may also be involved in the recruitment of monocytes into the arterial wall during artherosclerosis.

CCL14 (CC-1; CC-3; CKB1; MCIF; NCC2; SY14; HCC-1; HCC-3; NCC-2; SCYL2; SCYA14; FLJ16015; HCC-1(1-74); HCC-1/HCC-3)

The cytokine encoded by this gene induces changes in intracellular calcium concentration and enzyme release in monocytes. Multiple transcript variants encoding different isoforms were found for this gene. Read-through transcripts are also expressed that include exons from the upstream cytokine gene 15.

CCL15 (LKN1; NCC3; SY15; HCC-2; LKN-1; MIP-5; NCC-3; SCYL3; MIP-1D; MRP-2B; SCYA15; HMRP-2B)

The cytokine encoded by this gene is chemotactic for T cells and monocytes and induces N-acetyl-beta-D-glucosaminidase release in monocytes. It induces changes in intracellular calcium concentration in monocytes and is thought to act through the CCR1 receptor. Read-through transcripts are expressed that include exons from the downstream cytokine gene, chemokine (C-C motif) ligand 14.

CCL16 (LEC; LMC; CKb12; HCC-4; LCC-1; Mtn-1; NCC-4; SCYL4; ILINCK; SCYA16)

The cytokine encoded by this gene displays chemotactic activity for lymphocytes and monocytes but not for neutrophils. This cytokine also shows a potent myelosuppressive activity and suppresses proliferation of myeloid progenitor cells. The expression of this gene is up-regulated by IL-10.

CCL17 (TARC; ABCD-2; SCYA17; A-152E5.3)

The cytokine encoded by this gene displays chemotactic activity for T lymphocytes, but not monocytes or granulocytes. The product of this gene binds to chemokine receptors CCR4 and CCR8. This chemokine plays important roles in T cell development in thymus as well as in trafficking and activation of mature T cells. Since TARC expression could be detected in Hodgkin's lymphoblastoma and several other cancers, CCR4 chemoattractant activity seems to affect the course of some diseases specifically.

CCL18 (CKb7; PARC; AMAC1; DCCK1; MIP-4; AMAC-1; DC-CK1; SCYA18)

The cytokine encoded by this gene displays chemotactic activity for naive T cells, $CD4^+$ and $CD8^+$ T cells and nonactivated lymphocytes, but not for monocytes or granulocytes. This chemokine attracts naive T lymphocytes toward DCs and activated macrophages in lymph nodes. It may play a role in both humoral and cell-mediated immunity responses.

CCL19 (ELC; CKb11; MIP3B; MIP-3b; SCYA19)

The cytokine encoded by this gene may play a role in normal lymphocyte recirculation and homing. It also plays an important role in trafficking of T cells in thymus, and in T cell and B cell migration to secondary lymphoid organs. It specifically binds to chemokine receptor CCR7.

CCL20 (CKb4; LARC; ST38; MIP3A; MIP-3a; SCYA20)

CCR6/CCL20 signaling axis was shown to be involved in gut-associated lymphatic tissue formation and might be involved with nodular infiltrates in the kidney [168]. Through CCL20 function to direct IL-10 producing regulatory T cells to the colon this chemokine might be involved in pathogenesis of colitis and bowel diseases [169].

CCL21 (ECL; SLC; CKb9; TCA4; 6Ckine; SCYA21)

Similar to other chemokines the protein encoded by this gene inhibits hematopoiesis and stimulates chemotaxis. This protein is chemotactic in vitro for thymocytes and activated T cells, but not for B cells, macrophages, or neutrophils. The cytokine encoded by this gene may also play a role in mediating homing of lymphocytes to secondary lymphoid organs. It is a high affinity functional ligand for CCR7 that is expressed on T and B lymphocytes.

CCL22 (MDC; ABCD-1; SCYA22; STCP-1; DC/B-CK; A-152E5.1)

The cytokine encoded by this gene displays chemotactic activity for monocytes, DCs, natural killer cells and for chronically activated T lymphocytes. It also displays a mild activity for primary activated T lymphocytes and has no chemoattractant activity for neutrophils, eosinophils and resting T lymphocytes. The product of this gene binds to chemokine receptor CCR4. This chemokine may play a role in the trafficking of activated T lymphocytes to inflammatory sites and other aspects of activated T lymphocyte physiology.

CCL23 (CKb8; MIP3; Ckb-8; MIP-3; MPIF-1; SCYA23; Ckb-8-1; CK-BETA-8)

The cytokine encoded by this gene displays chemotactic activity on resting T lymphocytes and monocytes, lower activity on neutrophils and no activity on activated T lymphocytes. The protein is also a strong suppressor of colony formation by a multipotential hematopoietic progenitor cell line. In addition, the product of this gene is a potent agonist at CC chemokine receptor 1. Two alternatively spliced variants encoding different active isoforms were identified.

CCL24 (Ckb-6; MPIF2; MPIF-2; SCYA24)

The cytokine encoded by this gene displays chemotactic activity on resting T lymphocytes, a minimal activity on neutrophils, and is negative on monocytes and activated T lymphocytes. The protein is also a strong suppressor of colony formation by a multipotential hematopoietic progenitor cell line.

CCL25 (TECK; Ckb15; SCYA25)

The cytokine encoded by this gene displays chemotactic activity for DCs, thymocytes, and activated macrophages but is inactive on peripheral blood lymphocytes and neutrophils. The product of this gene binds to chemokine receptor CCR9.

CCL26 (IMAC; TSC-1; MIP-4a; SCYA26; MGC126714; MIP-4alpha)

The cytokine encoded by this gene displays chemotactic activity for normal peripheral blood eosinophils and basophils. The product of this gene is one of three related chemokines that specifically activate chemokine receptor CCR3. This chemokine may contribute to the eosinophil accumulation in atopic diseases.

CCL27 (ALP; ILC; CTAK; CTACK; PESKY; ESKINE; SCYA27)

The protein encoded by this gene is chemotactic for skin-associated memory T lymphocytes. This cytokine may also play a role in mediating homing of lymphocytes to cutaneous sites. It specifically binds to CCR10. Studies of a similar murine protein indicate that these protein-receptor interactions have a pivotal role in T cell-mediated skin inflammation.

CCL28 (MEC; CCK1; SCYA28)

The cytokine encoded by this gene displays chemotactic activity for resting CD4 or CD8 T cells and eosinophils. The product of this gene binds to chemokine receptors CCR3 and CCR10. This chemokine may play a role in the physiology of extracutaneous epithelial tissues, including diverse mucosal organs.

C-X-C-CHEMOKINES•

CXC chemokines are further subdivided into ELR and non-ELR types based on the presence or absence of a glu-leu-arg sequence adjacent and N terminal to the CXC motif. ELR types are chemotactic for neutrophils, while non-ELR types are chemotactic for lymphocytes.

CXCL1 (FSP; GRO1; GROa; melanoma growth stimulating activity (MGSA); NAP-3; SCYB1; MGSA-a)

The activity of the CXCR1 receptor induces the attraction of neutrophils and may be implicated through the adhesion of neutrophils to synovial microvessels in the development of arthritis.

CXCL2 (GRO2; GROb; MIP2; MIP2A; SCYB2; MGSA-b; MIP-2a; CINC-2a)

CXCL3 (GRO3; GROg; MIP2B; SCYB3; MIP-2b; CINC-2b)

CXCL4 (SCYB4; PF4)

Platelet factor-4 is a 70-amino acid protein that is released from the alpha-granules of activated platelets and binds with high affinity to heparin. Its major physiologic role appears to be neutralization of heparin-like molecules on the endothelial surface of blood vessels, thereby inhibiting local antithrombin III activity and promoting coagulation. As a strong chemoattractant for neutrophils and fibroblasts, PF4 probably has a role in inflammation and wound repair.

CXCL5 (SCYB5; ENA-78)

This chemokine is produced concomitantly with IL-8 in response to stimulation with either IL-1 or tumor necrosis factor-alpha (TNFa). This chemokine is a potent chemotaxin involved in neutrophil activation. In vascular vessels CXCL5 was shown to bind the non-signaling 7TM-GPCR DARC Duffy-antigen receptor for chemokines inhibiting the building of chemokine scavenging and gradients. DARC can be transcytosed and might be therefore involved in storage, degradation or transportation of chemokines. DARC binds rather unspecifically and has the highest affinity towards CXCL1, CXCL7, CXCL8, CCL2 and CCL5 [170].

CXCL6 (granulocyte chemotactic protein (GCP)2; CKA-3; GCP-2; SCYB6)

• supplied by OMIM or RefSeq

CXCL7 (PBP; TC1; TC2; TGB; LDGF; MDGF; TGB1; B-TG1; CTAP3; NAP-2; SCYB7; THBGB; LA-PF4; SCAR10; THBGB1; Beta-TG; CTAPIII; CTAP-III; pro-platelet basic protein(PPBP))

This growth factor is a potent chemoattractant and activator of neutrophils. It has been shown to stimulate various cellular processes including DNA synthesis, mitosis, glycolysis, intracellular cAMP accumulation, prostaglandin E2 secretion, and synthesis of hyaluronic acid and sulfated glycosaminoglycan. It also stimulates the formation and secretion of plasminogen activator by synovial cells.

CXCL8 (NAF; GCP1; LECT; LUCT; GCP-1; LYNAP; MDNCF; NAP-1; IL-8)

This chemokine is one of the major mediators of the inflammatory response. This chemokine is secreted by several cell types. It functions as a chemoattractant, and is also a potent angiogenic factor. This gene is believed to play a role in the pathogenesis of bronchiolitis, a common respiratory tract disease caused by viral infection.

CXCL9 (CMK; MIG; Humig; SCYB9; crg-10)

The function of this gene has not been specifically defined; however, it is thought to be involved in T cell trafficking.

CXCL10 (C7; IFI10; INP10; IP-10; crg-2; mob-1; SCYB10; gIP-10)

This chemokine is a ligand for the receptor CXCR3. Binding of this protein to CXCR3 results in pleiotropic effects, including stimulation of monocytes, natural killer and T-cell migration, and modulation of adhesion molecule expression.

CXCL11 (IP9; H174; IP-9; b-R1; I-TAC; SCYB11; SCYB9B)

This protein induces a chemotactic response in activated T-cells and is the dominant ligand for CXCR3. The gene encoding this protein contains 4 exons and at least three polyadenylation signals which might reflect cell-specific regulation of expression. IFN-gamma is a potent inducer of transcription of this gene.

CXCL12 (IRH; PBSF; SDF1; TLSF; SDF1A; SDF1B; TPAR1; SCYB12)

This is a stromal cell-derived alpha chemokine member of the intercrine family. Its receptor CXCR4 can activate lymphocytes and were implicated in the metastasis of some cancers such as breast cancer. Mutations in this gene are associated with resistance to human immunodeficiency virus type 1 infections. Multiple transcript variants encoding different isoforms were found for this gene. Since CXCR7 is an alternative receptor for CXCR4 ligands it was also shown to play critical roles in CXCR4 mediated diseases like rheumatoid arthritis and can therefore be considered as additional target for treatments. CXCR7 was also found to be expressed in various types of cancer cells. It signals via AKT and MAPK kinases and might induce migration in some cancer cells upon ITAC-mediated stimulation.

CXCL13 (BLC; BCA1; ANGIE; BCA-1; BLR1L; ANGIE2; SCYB13)

B lymphocyte chemoattractant is strongly expressed in the follicles of the spleen, lymph nodes, and Peyer's patches. It preferentially promotes the migration of B lymphocytes (compared to T cells and macrophages), apparently by stimulating calcium influx into, and chemotaxis of, cells expressing Burkitt's lymphoma receptor 1 (BLR-1). It may therefore function in the homing of B lymphocytes to follicles.

CXCL14 (KEC; KS1; BMAC; BRAK; NJAC; MIP2G; MIP-2g; SCYB14)

This cytokine displays chemotactic activity for monocytes but not for lymphocytes, DCs, neutrophils or macrophages. It has been implicated that this cytokine is involved in the homeostasis of monocyte-derived macrophages rather than in inflammation.

CXCL15 (WECHE; SCYB15; LUNGKINE)

CXCL16 (SRPSOX; CXCLG16; SR-PSOX)

CXCL17 (DMC; VCC1; DCIP1; VCC-1)

CXCL18

CXCL19 (SCL)

CXCL20

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