

Th9 and other IL-9-producing cells in allergic asthma

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Abstract Allergic asthma is a worldwide increasing chronic disease of the airways which affects more than 300 million people. It is associated with increased IgE, mast cell activation, airway hyperresponsiveness (AHR), mucus overproduction and remodeling of the airways. Previously, this pathological trait has been associated with T helper type 2 (Th2) cells. Recently, different CD4⁺ T cell subsets (Th17, Th9) as well as cells of innate immunity, like mast cells and innate lymphoid cells type 2 (ILC2s), which are all capable of producing the rediscovered cytokine IL-9, are known to contribute to this disease. Regarding Th9 cells, it is known that naïve T cells develop into IL-9-producing cells in the presence of interleukin-4 (IL-4) and transforming growth factor beta (TGFβ). Downstream of IL-4, several transcription factors like signal transducer and activator of transcription 6 (STAT6), interferon regulatory factor 4 (IRF4), GATA binding protein 3 (GATA3), basic leucine zipper transcription factor, ATF-like (BATF) and nuclear factor of activated T cells (NFAT) are activated. Additionally, the transcription factor PU.1, which is downstream of TGFβ signaling, also seems to be crucial in the development of Th9 cells. IL-9 is a pleiotropic cytokine that influences

various distinct functions of different target cells such as T cells, B cells, mast cells and airway epithelial cells by activating STAT1, STAT3 and STAT5. Because of its pleiotropic functions, IL-9 has been demonstrated to be involved in several diseases, such as cancer, autoimmunity and other pathogen-mediated immune-regulated diseases. In this review, we focus on the role of Th9 and IL-9-producing cells in allergic asthma.

Keywords Th9 · IL-9 · Allergic asthma · IL-9R · Mast cells · ILC2

Allergic asthma

Asthma is a common chronic inflammatory disease of the airways involving cells of the innate and adaptive immune system. About 300 million people worldwide are affected by the disease. Two forms of asthma have been clinically defined, non-allergic (or intrinsic) asthma and allergic (atopic) asthma. Allergic asthma mostly affects children and nearly 50% of adult asthma patients and is triggered by proteins of common inhaled or ingested allergens such as house dust mite, animal dander or plant and tree pollen. The onset of intrinsic asthma usually occurs later in life and is caused not by allergens but by viral infections of the lower airways or irritants, e.g. tobacco smoke, cold air or stress. Often in patients suffering from asthma, components of both the intrinsic and the extrinsic type can be observed [1–3].

Typically, asthma is characterized by airway hyperresponsiveness (AHR), reversible airway obstruction, mucus overproduction, airway remodeling and high serum IgE levels. These symptoms lead to a narrowing of the airways and cause repeated periods of shortness of breath, wheezing and chest tightness, mostly because of mast cell activation

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followed by histamine release and other bronchoconstrictors, such as proteases, upon IgE cross-linking [2, 3].

In association with the airway epithelium and airway mucosa, dendritic cells (DCs) take up allergens, process them into small peptides and present them to naïve T cells in the lymph nodes. The interaction of DCs with naïve T cells and the presence of interleukin-4 (IL-4) lead to T helper type 2 (Th2) cell differentiation and migration into the mucosa. Th2 cells start to secrete a cluster of cytokines including IL-3, IL-4, IL-5, IL-9, IL-13 and granulocyte macrophage colony-stimulating factor (GM-CSF). These cytokines orchestrate the inflammatory response that takes place in asthma. This response includes eosinophil maturation, migration and survival which are triggered by IL-3, IL-5 and GM-CSF and recruitment of basophils promoted by IL-3 and GM-CSF. Furthermore, IL-4 stimulates immunoglobulin class-switch to IgE in B cells, while IL-3, IL-9 and IL-13 facilitate the differentiation and maturation of mast cells. After allergen challenge, eosinophils and mast cells secrete pro-inflammatory mediators such as histamine, leukotrienes, cytokines and chemokines that enhance the typical pathological features of asthma as mentioned before [2, 3].

Th9 cell development and signaling

Th9 cells are a subset of T cells that develop from naïve T cells in the presence of IL-4 and transforming growth factor beta (TGF β) [4]. Besides these two factors, several other transcription factors seem to play a role in the development of these cells. Signal transducer and activator of transcription 6 (STAT6) is the major component of the IL-4 receptor (IL-4R) signaling pathway and is therefore also involved in controlling the Th9 phenotype [5]. It is known that STAT6 is able to suppress the Th1-specific transcription factor T-box expressed in T cells (T-bet) and the TGF β -induced expression of forkhead box P3 (FoxP3), which are both known to inhibit IL-9 production [5–7]. GATA binding protein 3 (GATA3), the main transcription factor of Th2 cells, is another important target of STAT6 and is expressed in Th9 cells as well, but to a lower extent than in Th2 cells [5–7]. Therefore, it might also play a role in the differentiation of Th9 cells. However, its exact function remains unclear, because of inconsistent results [5, 7, 8]. Additionally, the transcription factor PU.1 (also known as Spi-1 proto-oncogene (SPI1)), which is downstream of TGF β signaling, seems to be crucial in the development of Th9 cells [9, 10]. PU.1 belongs to the ETS family of transcription factors and on the one hand represses the development of Th2 cells and on the other hand it binds to the *Il9* promoter to enhance its expression [9, 11, 12]. Interferon regulatory factor 4 (IRF4) is another factor involved in Th9 cell differentiation but is also known to be expressed in Th2 and Th17 cells [13, 14]. T cells deficient in IRF4 show a defective Th9 development [15]. IRF4 expression as well as its ability to directly

bind to the *Il9* promoter is STAT6 dependent [5]. IRF4 is known to build a complex with the basic leucine zipper transcription factor, ATF-like (BATF) as well as with Jun to induce Th17 cell development, and there seems to be a similar transcriptional requirement in Th9 cells. BATF and IRF4 cooperate in binding and thereby activate the *Il9* locus. In contrast to IRF4, BATF deficiency resulted in a marked defect in the expression of nearly all Th9-associated genes [16]. It has also been demonstrated that T cell receptor (TCR) and co-stimulatory molecules have an impact in IL-9 production as well. On the one hand, TCR signaling and CD28-mediated co-stimulation lead to the activation of nuclear factor of activated T cells (NFAT) and on the other hand, the TCR and OX40 (also known as tumour necrosis factor receptor superfamily, member 4 (TNFRSF4)) co-stimulation activates nuclear factor- κ B (NF- κ B) [17, 18]. Previous studies showed that NFAT1 deficiency resulted in greatly reduced amounts of IL-9 production [17]. Thus, many different transcription factors that are also known to play a role in the pathogenesis of allergic asthma are also involved in the differentiation and regulation of Th9 cell development and IL-9 production. In the following paragraphs, we will summarize the current knowledge of IL-9-producing cells in asthma as well as factors inducing or inhibiting their differentiation and function and review the effects of IL-9 on target cells in asthma.

Th9 and IL-9-producing cells in allergic asthma

Th9 cells mainly produce the cytokine IL-9 but are also known to secrete the cytokines IL-10 and IL-21 [6, 9, 19]. Because of its pleiotropic effects, IL-9 can influence a variety of distinct cell types like, e.g. T cells, B cells, mast cells and airway epithelial cells. Furthermore, it is produced by different immune cells that are involved in the immunopathology of allergic asthma. Among those cells are other T cell subsets, mast cells and innate lymphoid cells type 2 (ILC2s) [9, 20–23].

Th9 cells

Th9 cells are a distinct helper T cell subset that secretes IL-9 as a signature cytokine. Previously published studies could demonstrate that in murine models of asthma, Th9 cells are found in the draining lymph nodes and respiratory tract, especially during early phases of the disease [24–26]. Furthermore, Th9 cells were the main *in vivo* source of IL-9. The addition of the pro-allergic cytokines IL-25 and thymic stromal lymphopoietin (TSLP) in experimental asthma models lead to an increase in Th9 cells and IL-9 production. Moreover, neutralizing TGF β and activin A to inhibit Th9 cell differentiation *in vivo* resulted in an impaired development of allergic diseases. Conversely, Th9 cells *in vitro* generated with either

TGF β or activin A and transferred to recipient mice were sufficient to induce airway inflammation characterized by eosinophil recruitment, increased mast cell numbers and serum IgE levels [24]. Furthermore, transgenic expression of IL-9 is sufficient in itself to cause bronchial hyperresponsiveness through its effects on the respiratory epithelium and the enhanced release of Th2 cytokines [27–29]. Additionally, mice with conditional deficiencies in IRF4 or PU.1 or germ line deficiencies in BATF have a decreased inflammation [12, 13, 16, 30–32]. Transfer of DO11.10 Th9 cells to recipient mice resulted in allergic airway disease after treatment with ovalbumin (OVA). Furthermore, when these recipient mice were additionally treated with an anti-IL-9 antibody, many of the inflammatory features were blocked [13]. Th9 cells mediate disease with the production of IL-9, which promotes mast cell and eosinophil accumulation, Th2 cytokine production, AHR and mucus hypersecretion.

Beside the observations in murine asthma models, enhanced expressions of IL-9 as well as IL-9 receptor (IL-9R) have been found in the lungs of asthmatic patients even before Th9 cells were described as a distinct T helper cell subset. Furthermore, single nucleotide polymorphisms (SNPs) in the genes encoding *IL9R* and *IL9* have been associated with beneficial outcomes in allergen sensitization, especially in male subjects [33]. In vitro studies using peripheral blood mononuclear cells (PBMCs) isolated from atopic and non-atopic infants revealed increased IL-9 secretion, in both response to unspecific as well as allergen-specific stimulation of the cells. In addition, in vitro differentiation of naïve T cells into Th9 cells led to elevated IL-9 secretion, *SP11* (encodes for PU.1) messenger RNA (mRNA) expression and STAT6 phosphorylation in atopic infants, as compared to non-atopic infants [34]. In another study, PBMCs were isolated from adult atopic and healthy control subjects to directly quantify Th9 cells by flow cytometry. Here, they observed that in atopic patients, Th9 cells are more abundant than in the healthy controls, which correlated with plasma IgE levels [24]. Together, these data indicate an important role for Th9 cells in atopic diseases such as allergic asthma not only in murine models but in human disease as well and open new possibilities to create novel therapeutic strategies. In murine models, the administration of anti-IL-9 antibodies followed by allergen exposure showed beneficial effects, such as decreased expression of the three Th2-associated cytokines IL-4, IL-5 and IL-13; decreased number of eosinophils in the lungs and decreased AHR [35]. However, in a large human trial with patients with uncontrolled moderate-to-severe asthma, treatment with the humanized IL-9-neutralizing antibody MEDI-528 (also called enokizumab) in addition to current controller medication did not improve the FEV1 values, the asthma exacerbation rate or the health-related quality of life [36]. The limited success of such trials was also previously observed, when other important cytokines, e.g. IL-5 or IL-13, were blocked. It is assumed

that this is due to the heterogeneity of asthma, as the underlying immune response varies from patient to patient [37]. In the future, by using personalized medicine, it might be possible, however, to first determine the predominant cell types or cytokine patterns in a person suffering from asthma and then try to inhibit the effector cytokines respectively. Using this approach might identify a subgroup of patients with IL-9-mediated asthma, in which the IL-9-neutralizing antibody might be beneficial.

Interestingly, the tick-derived saliva protein sialostatin L has been shown to reduce IL-9 derived from Th9 cells as well as from mast cells in murine models of asthma. Furthermore, this cysteine protease inhibitor was reported to diminish maturation and activation of DCs and CD4⁺ T cell expansion, indicating a broader inhibition of key cell types involved in asthma pathogenesis [38].

Other Th subsets

In addition to Th9 cells, other CD4⁺ T cell subsets, such as Th2, Th17 and Treg cells, have been shown to express IL-9, but the amount of IL-9 they produce, compared to Th9 cells, is lower [9, 21, 39].

Th2 cells are the classical cell type that is described to promote allergic inflammation and cardinal features of allergic asthma. IL-9 was first associated with Th2 cells that produce IL-4, IL-5 and IL-13 in models of Th2-associated immunity. *Leishmania major*-infected BALB/c mice, which generate a Th2 immune response, displayed high levels of IL-9 expression in isolated T cells. Additionally, in this study, they found that those T cells were the main producers of IL-9 in vivo and IL-9 expression correlated with the expansion of Th2 cell populations. Furthermore, IL-9 production could be suppressed by IL-4 blockade [40], but it was unclear whether the correlation between IL-9 production and Th2 cells is due to a direct secretion of IL-9 by Th2 cells or if IL-4 derived from Th2 cells supports the development of Th9 cells from naïve T cells or Th2 cells. Previous studies demonstrated that fully differentiated Th2 cells subsequently produce IL-9 when they are cultured in the presence of TGF β [7].

Th17 cells are also known to play a role in the pathogenesis of allergic asthma. After sensitization with allergens, Th17-mediated immune responses lead to neutrophilia in the airways, acute AHR and mucus overproduction [41–43]. In asthmatic patients IL-17A, the main cytokine produced by these cells, was found induced compared to healthy controls [44–46]. Furthermore, Th17 cells have been shown to enhance the antigen-induced, Th2-mediated recruitment of eosinophils to the airways [47]. It has been demonstrated that in vivo differentiated Th17 cells, which express retinoic acid receptor-related orphan receptor- γ t (ROR γ t), are able to produce IL-9 after restimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin [21]. Additionally, during

in vitro Th17 cell differentiation, a co-expression of IL-17 and IL-9 has also been observed. The IL-9 secretion by Th17 cells seems to be regulated by IL-23, which was shown to inhibit the expression of IL-9 [20]. IL-23 is an important factor in the maintenance and expansion of Th17 cells after their initial differentiation [48, 49]. In Th17 cells, IL-9 seems to function as an autocrine growth factor that promotes Th17 cell differentiation [20, 21].

Treg cells play an important role in allergic asthma because they are able to suppress effector cells. In previous studies, it was shown that Treg cells prevent AHR- and Th2-mediated inflammation of the airways in an IL-10-dependent way [50–52]. Additionally, in human studies, lower numbers of CD4⁺CD25⁺ Treg cells as well as lower expression levels of *FOXP3* mRNA have been observed [53]. The main transcription factor of Treg cells is FoxP3, which is induced by TGF β . FoxP3 is an antagonist of ROR γ t and therefore able to inhibit Th17 cell differentiation [54]. Regarding IL-9 expression by Treg cells, there are inconsistent reports. Some murine data showed that co-expression of IL-9 and FoxP3 can occur in in vitro purified Treg cells as well as in Treg cells found in tolerant allografts in vivo [21, 39, 55]. Other groups could not show co-expression of IL-9 and FoxP3 in in vitro experiments [7, 20].

Concerning CD4⁺ T cell subsets, it seems that TGF β is the shared factor that, alone or in combination with other cytokines, induces IL-9 expression during Treg, Th9 and Th17 cell differentiations. Additionally, TGF β also promotes IL-9 production in already differentiated Th2 cells [7].

Innate lymphoid cells

ILC2s are a rare cell population that do not express any of the known lineage markers and respond to IL-25 and IL-33. They express ST2, an orphan member of the IL-1 receptor family [56]. ILC2s are known to be important producers of the Th2-associated cytokines IL-5 and IL-13. Therefore, they seem to play a relevant role in the pathogenesis of allergic asthma, since it could be demonstrated that ILC2s increase after allergen exposure [56–59]. In a model of papain-induced airway inflammation, IL-9-producing ILC2s are induced. Furthermore, they were the main source of IL-9 and its production was transient and IL-2 dependent. Additionally, IL-9 enhanced the production of IL-5 and IL-13 in ILC2s [23]. Moreover, another group could demonstrate that IL-9 is a crucial autocrine amplifier of ILC2 function and survival [60].

Mast cells

Mast cells are widely distributed throughout vascularized tissues, particularly near surfaces exposed to the external environment, including the airways. They are critical effector cells in IgE-associated allergic diseases like asthma. Mast cells can be activated by the cross-linking of IgE, which is bound to the

Fc ϵ RI on their surface, after antigen exposure. After their activation, they degranulate and release pro-inflammatory mediators such as histamine, proteases and cytokines. IL-9 has mainly been described to target mast cells, but it has also been reported to be produced by these cells. Its production seems to occur in an autocrine manner as a consequence of IgE cross-linking and as response to IL-9-induced signals. After degranulation, mast cells release, among others, histamine and IL-1 β which both can promote further production of IL-9 [22, 61]. IL-9 seems to act as a growth factor for mast cells, therefore promoting their expansion and survival during acute immune responses.

Natural killer T cells

Natural killer T (NKT) cells are a distinct subset of lymphocytes that are abundant in primary and secondary lymphoid organs as well as in the lung. They express surface markers that are characteristic of NK cells, e.g. NK1.1 and of conventional T cells, e.g. TCRs. NKT cells are selected by the non-polymorphic MHC class I-like protein CD1d and express a semi-invariant TCR (iTCR), consisting of, e.g. V α 14-J α 18 in mice and V α 24-J α 18 in humans, which make them less diverse than conventional T cells [62–64]. It has been proposed that they produce large amounts of IL-4 when they are stimulated by glycolipid ligands [65]. Additionally, in a murine model of allergic asthma, it has been demonstrated that iV α 14 NKT cells are required for airway eosinophilia, AHR, elevated IgE levels and Th2 cytokine production [66]. Previous studies showed that NKT cells are able to produce IL-9 when they are stimulated with IL-2. These IL-9-producing NKT cells also co-expressed the Th2-associated cytokines IL-4, IL-5 and IL-13 [67]. In a murine model of allergic airway inflammation, deficiency of CD1d-restricted NKT cells correlated with decreased IL-9 production. These findings were accompanied with reduced recruitment of mast cells to the lung [66]. Furthermore, in a report describing an IL-9-secreting NKT cell subset, called NKT9 cells, it was observed that these cells develop in the periphery upon stimulation with IL-4 and TGF β . Comparable to findings in T cells, mice deficient for IRF4 or BATF were observed to have a profound defect in developing NKT9 cells. Additionally, intratracheal transfer of IL-9⁺ enriched NKT cells into wild-type mice with subsequent intranasal (i.n.) challenge with house dust mite (HDM) extract and TSLP enhanced airway inflammation, while neutralizing IL-9 with an antibody in vivo, reduced eosinophil recruitment in the same setting [68]. Thus, NKT cells are able to promote in vivo IL-9 responses.

Activating factors of IL-9 expression

Previously published studies revealed that many different factors are positively or negatively influencing the expression of

IL-9 in different cell types, which is explained in detail in the following sections and shown in Fig. 1 and Table 1.

The Th9 cell-inducing cytokine TGF β is known to signal via Smad transcription factors, and it has been shown that Smad2 and Smad3 are important for *Ii9* transcription. Interestingly, Smad2/Smad3-mediated *Ii9* expression was shown to be dependent on IRF4 (see below), as these factors physically interact to bind to the *Ii9* promoter. Consistently, lack of both Smad2 and Smad3 in T cells protected mice from OVA-induced asthma with reduced airway inflammation, mucus production and IL-9 levels in the serum [8].

The established roles of factors such as STAT6 and PU.1 in the differentiation of Th9 cells can also be observed in allergic asthma. At a steady state, STAT6 phosphorylation and PU.1 expression were observed to be elevated in patients suffering from allergic asthma, as compared to healthy controls. Furthermore, after bronchial allergen challenge, STAT6 activation, as determined by phosphorylation status and PU.1, was further enhanced in asthmatic patients, while there was no effect in the control group [77].

In a mouse model of allergic asthma, IRF4 was identified as a crucial transcription factor for Th9 differentiation, as mice

deficient for this factor fail to develop this cell type. This finding was reinforced by the observations that knockdown of IRF4 using siRNA reduced IL-9 secretion from CD4⁺ T cells and that IRF4 is able to bind directly to the promoter region of *Ii9*, as revealed by using chromatin immunoprecipitation (ChIP) [13].

Recently, it has been shown that IRF4 can cooperate with the AP-1 transcription factor BATF in differentiated Th17 cells and that this complex binds to AP-1-IRF4 composite elements (AICEs) [85]. A further study now demonstrated that complementation of BATF in developing Th9 cells deficient for BATF or IRF4 moderately increased IL-9 production, while IRF4 complementation in these cells had a minor effect. When both factors were transduced together, however, a strong induction of IL-9 was observed, indicating a synergy between IRF4 and BATF [16]. Thus, beside its described prominent role in the differentiation of Th17 and Th2 cells, BATF also has a major impact on Th9 development. Furthermore, it has been shown that BATF-deficient mice display reduced features of experimental asthma in different murine models. There, in a classically ovalbumin-induced one, IL-9 levels in the

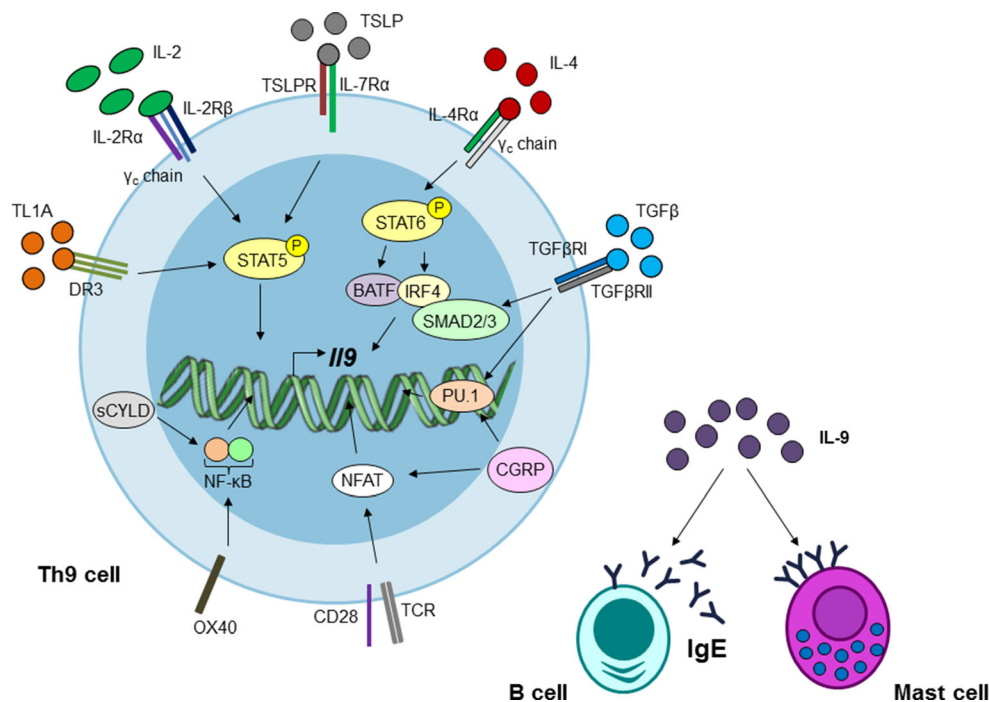


Fig. 1 Extracellular signals and intracellular pathways regulating Th9 cell differentiation. Beside the well-characterized IL-4/TGF β pathway, also a STAT5-dependent one has been proposed, where IL-2, TSLP or TL1A lead to STAT5 phosphorylation, with subsequent binding of STAT5 to the *Ii9* promoter. Furthermore, TCR stimulation leads to activation of NFAT, which can also bind to the *Ii9* promoter. The co-stimulatory molecule OX40 and the de-ubiquitinating enzyme sCYLD activate NF- κ B to bind to the *Ii9* promoter. IL-4 is known to induce STAT6 phosphorylation, which in turn can activate BATF and IRF4 expression to induce *Ii9* transcription, while TGF β signals via SMAD2/3, which interacts with IRF4 to induce IL-9. PU.1 is also activated by

TGF β and CGRP and augments IL-9 expression. CGRP has also been described to enhance NFAT binding to the *Ii9* promoter. The secreted IL-9 influences, e.g. B cells, where it increases IgE production and memory B cell differentiation, while it acts on mast cells as a growth factor and stimulates their effector function. TGF β transforming growth factor β , STAT signal transducer and activator of transcription, TSLP thymic stromal lymphopoietin, NFAT nuclear factor of activated T cells, NF- κ B nuclear factor- κ B, BATF basic leucine zipper transcription factor, ATF-like, IRF4 interferon regulatory factor 4, CGRP calcitonin gene-related peptide

Table 1 Molecules linked to IL-9 production and allergic asthma

Factor	Pathway/mechanism	Influence on Th9 cells	Role in asthma pathogenesis	Referenced in
Cytokines				
IL-4	Induces STAT6 phosphorylation	↑	Drives Th2 differentiation and IgE class-switch	[2–5]
TGF-β	Induces SMAD2/3 and PU.1	↑	Together with IL-6, it induces Th17 differentiation	[8]
TSLP	Induces STAT5 phosphorylation	↑	Contributes to airway inflammation and AHR and promotes Th2 differentiation via DCs	[32, 69, 70]
Co-stimulatory molecules				
OX-40	Ligation of OX-40 induces NF-κB p52-RelB subunit binding to the <i>Il9</i> promoter via TRAF6	↑	Supports Th1 and Th2 responses, inhibits FoxP3 ⁺ Treg cells	[18]
PD-L2	Suppression of TGFβ	↓	Influences inflammation and mucus production	[26]
TL1A	Signaling via DR3 induces STAT5 phosphorylation, which is able to bind to the <i>Il9</i> promoter	↑	Inhibition of Th9 cells in a model of chronic airway disease	[71]
Signaling molecules				
Itk	Engagement of IL-2/STAT5 pathway to induce IL-9 expression Lack of Itk reduces IRF4 expression	↑	Promotion of allergic airway disease in OVA- and papain-induced models	[72]
Tyk2	Inhibition of <i>IRF4</i> and <i>SP11</i> mRNA expressions	↓	Itk-enhancing SNPs observed in patients with asthma	[73]
Transcription factors				
BATF	Interacts with IRF4 to bind to the <i>Il9</i> promoter	↑	Positive modulation of AHR, eosinophilia and mucus production	[16, 31]
IRF4	Interacts with BATF to bind to the <i>Il9</i> promoter	↑	Dampens eosinophilia and high serum IgE levels and reduces Th2 and Th9 responses	[13, 16]
NFATc1	Cooperates with IRF4 to bind to the <i>Il9</i> promoter	↑	IRF4 deficiency protects from experimental asthma	[74, 75]
NFATc2	Binding to <i>Il9</i> promoter induces activating chromatin configuration to allow access for NF-κB subunit p65 and coactivator protein p300	↑	Involvement in Th2 and Th17 cell development and IgE class-switch	[17, 76]
NF-κB	NF-κB subunits p65 and p52-RelB are able to bind to the <i>Il9</i> promoter	↑	Inhibition of Th1 cells	[17, 76]
PU.1	Downstream of TGFβ signaling, which is able to bind to the <i>Il9</i> promoter	↑	Long-lived CD8 ⁺ memory cells expressing NFATc2 limit Th2 and Th17 development	[17, 76]
SMAD2/3	Interact with IRF4 to promote <i>Il9</i> expression	↑	Induces pro-inflammatory molecules, e.g. TNFα, IL-6, IL-8, eotaxin, COX-2, IL-2R, ICAM-1 and VCAM-1	[17, 18, 77, 78]
STAT6	Phosphorylation induces expressions of BATF and IRF4	↑	Represses Th2 development and enhances IL-9 expression	[9–12, 77]
Other molecules				
Cbl-b	Ubiquitination of STAT6	↓	Influences airway inflammation and mucus production	[8]
CGRP/cAMP/PKA	PU.1 induction via PKA, inhibition of GSK-3b and enhanced NFATc2 binding to the <i>Il9</i> promoter	↑	Elevated phosphorylation status in patients with asthma	[5, 77]
COX-2	COX-2-derived prostaglandins PGE2 and PGD2 suppress Th9 development	↓	Negatively regulates Th2 and Th9 responses	[79]
CYLD	Short isoform sCYLD enhances NF-κB activity	↑	Promotion of airway inflammation	[80]
SIRT1	Histone deacetylase, modulates stress responses, energy metabolism and inflammatory processes	↓	Inhibits Th9 development in allergic asthma	[81]
VitD	VitD and dexamethasone reduce IL-9 production in memory CD4 ⁺ T cells	↓	Influence on different other T cell subsets	[82]

STAT signal transducer and activator of transcription, *TGFβ* transforming growth factor β, *TSLP* thymic stromal lymphopoietin, *AHR* airway hyperresponsiveness, *NF-κB* nuclear factor-κB, *FoxP3* forkhead box P3, *PD-L2* programmed death ligand 2, *IRF4* interferon regulatory factor 4, *SNP* single nucleotide polymorphism, *Tyk2* tyrosine kinase 2, *BATF* basic leucine zipper transcription factor ATF-like, *NFAT* nuclear factor of activated T cells, *TNFα* tumour necrosis factor α, *Cox-2* cyclooxygenase 2, *ICAM-1* intercellular adhesion molecule 1, *VCAM-1* vascular cell adhesion molecule 1, *CGRP* calcitonin gene-related peptide, *PKA* protein kinase A, *GSK-3b* glycogen synthase kinase-3b, *VitD* vitamin D

bronchoalveolar lavage (BALF) were found decreased in the absence of BATF. Using adoptive transfer of wild-type

or BATF^{−/−} CD4⁺ T cells into immunocompromised hosts, followed by an IL-9/Th9-dependent asthma model (i.n.

challenge with OVA and TSLP, see below), revealed reduced airway inflammation and mucus production when BATF-deficient cells were transferred [16, 31].

It has been demonstrated that members of the NFAT family of transcription factors are involved in the pathogenesis of allergic asthma, where they seem to have distinct effects. Mice deficient in NFATc2 (also known as NFAT1) show an increased AHR, remodeling in the lungs and higher serum IgE levels when exposed to OVA. These observations were dependent on NFATc2 expression in long-lived memory CD8⁺ T cells, which controlled the production of IL-2 and interferon gamma (IFN γ) in lung CD8⁺ T cells, thereby limiting the development of Th2 and Th17 cells in the lungs of allergen treated mice [76]. Previous reports also demonstrated a potential role for NFATc2 in the production of IL-9. Binding of NFATc2 to the *Il9* promoter induced a transcriptionally active chromatin configuration that created access for the p65 subunit of NF- κ B and the co-activator protein p300 to the *Il9* promoter resulting in induced IL-9 production [17]. In contrast to NFATc2, mice lacking NFATc1 (also known as NFAT2) in CD4⁺ T cells exhibited a significant reduction in lung Th2 and Th17 cells as well as an increase of Th1 cells when exposed to OVA. Additionally, BATF was found decreased in the lungs of these mice and serum OVA-specific IgE and IgG1 levels were significantly decreased [74]. Recently, we have shown that NFATc1 as well as IRF4 are upregulated in PBMCs of allergic asthmatic children. Both factors are able to cooperate and seem to be involved in IL-9 production in these cells, since an upregulation of IL-9 in the supernatants of cultured PBMCs was observed [75].

Recently, it has been demonstrated that calcitonin gene-related peptide (CGRP), which is a physiological mediator derived from the nervous system and able to activate cAMP/protein kinase A (PKA), promotes IL-9 production. The CGRP and the cAMP/PKA pathway are known to be important regulators in several immune responses and allergic diseases. Moreover, this study revealed that PKA inhibits glycogen synthase kinase (GSK)-3 β function and enhances NFATc2 binding to the *Il9* promoter region as a new regulatory mechanism in Th cells. Additionally, the CGRP/cAMP/PKA pathway also induces the expression of PU.1, a critical regulator of Th9 cell differentiation, via PKA and independently of NFATc2 [80].

OX40 is a co-stimulatory protein expressed on T cells, belonging to the TNFR superfamily and known to engage the NF- κ B pathway. While the co-stimulation via OX40 in T cells favours their proliferation and survival, it has been shown to support Th1 and Th2 responses respective to the surrounding cytokine milieu and to inhibit FoxP3⁺ T cells. Recently, also, OX40-mediated induction of Th9 differentiation was reported. In this study, it was shown that ligation of OX40 led to enhanced in vitro Th9 differentiation in a PU.1-independent manner. Here, it was observed that in addition to the above-mentioned binding of the p65 subunit of NF- κ B to

the *Il9* promoter [17], also, the p52-RelB subunit is able to directly bind to the *Il9* promoter and stimulate *Il9* expression after ligation of OX40, while the p50-RelA subunit did not bind to the promoter. Further studies revealed that this pathway is dependent on the adaptor molecule and ubiquitin ligase TRAF6, upstream of NF- κ B-inducing kinase (NIK). In an OVA model of allergic asthma, a role of OX40 for inflammation, mucus secretion and IL-9 production was confirmed by comparing OX40-deficient mice to wild-type mice [18].

These findings are further supported by data obtained from the examination of transgenic mice with a T cell-specific overexpression of a short isoform of the de-ubiquitinating enzyme CYLD (CYLD^{ex7/8}xCD4^{cre} mice). Full-length CYLD is known to de-ubiquitinate signaling molecules such as TRAF6 or NEMO, facilitating a downregulation of NF- κ B activity, while the short isoform (sCYLD) is associated with enhanced NF- κ B activity. In in vitro differentiated Th9 cells overexpressing sCYLD, substantially higher IL-9 secretion was observed, while IL-4 secretion from in vitro generated Th2 cells did not differ, as compared to wild-type cells. Furthermore, in an adjuvant-free OVA-induced model of allergic asthma using CYLD^{ex7/8}xCD4^{cre} mice, it could be shown that in vitro restimulation with OVA induced IL-9 secretion from total lung cells, while IL-4, IL-5 and IL-13 release was not significantly altered when compared to the wild-type setting. Other features of allergic asthma, such as airway inflammation, mucus production and serum IgE, were concomitantly found to be augmented when sCYLD was overexpressed. Interestingly, i.n. administration of an IL-9-neutralizing antibody during the challenge phase of the asthma protocol reversed the increased inflammation and serum IgE, indicating an IL-9-dependent mechanism in CYLD^{ex7/8}xCD4^{cre} mice [82].

Another co-stimulatory molecule described to have a contributing role in experimental allergic airway disease is the cytokine TL1A. It is a member of the TNF family (TNFSF15) and mediates signals to T cells and ILCs via its receptor DR3 (TNFRSF25). Both blockade of TL1A and DR3 deficiency have been shown to protect from allergic airway disease, and recently, these findings were extended by the observation that in the absence of DR3, IL-9 expression was reduced in an OVA- as well as a papain-induced model. TL1A was further able to enhance Th9 differentiation from naïve T cells via DR3 and dependent on TGF β while being independent of STAT6. Further studies revealed that beside the previously described IL-4-TGF β pathway, also a distinct IL-2-STAT5 pathway is able to induce Th9 differentiation. Here, the proposed mechanism involved increased STAT5 activation through TL1A and DR3, with subsequent binding of STAT5 to the *Il9* promoter [71].

In line with these observations, another group reported that the pleiotropic cytokine TSLP is able to increase IL-9 production in Th9 cells via direct activation of STAT5 with its binding to the *Il9* promoter. Furthermore, they showed here that

TSLP is able to mediate allergic airway inflammation by increasing the Th9 response [32]. TSLP has been implicated to have a broad impact in the development of allergic asthma, as overexpression of TSLP in the lungs leads to the development of severe airway inflammation and AHR [69]. TSLP also acts directly on DCs to promote their maturation and the expression of co-stimulatory factors like OX40 ligand to enhance the priming of naïve CD4⁺ T cells towards a pro-inflammatory Th2 phenotype [70]. It has been recently shown that myeloid DCs from allergic asthma patients pulsed with TSLP were able to promote the differentiation of Th9 cells in a TGFβ-dependent way [86].

Beside the stimulation with different cytokines and through co-stimulatory molecules, TCR signaling is also of utter importance for the differentiation of T cell subsets. The Tec family tyrosine kinase Itk is involved in the distal transmission of TCR signals, and it has been shown to have a modulatory, rather than an essential, role in this process. However, lack of Itk is associated with reduced phospholipase C gamma (PLCγ) activation and subsequently decreased activation of, e.g. NFAT or MAP kinases. Furthermore, Itk was described to balance Th17 versus Treg differentiation and its deficiency was implicated in deficient Th2 responses. For asthma, Itk-enhancing polymorphisms have been correlated with disease incidence. A recent study analyzing Itk-deficient naïve CD4⁺ T cells under Th9 polarizing conditions showed a defect in IL-9 production along with reduced IRF4 expression. This study further revealed that IL-9 expression was not rescued by NFAT overexpression but by engaging the IL-2-STAT5 pathway. By adding IL-2 to the culture medium, Itk-deficient cells were able to produce high amounts of IL-9 and express IRF4 in levels comparable to wild-type cells. Furthermore, transducing Itk^{-/-} cells with a vector expressing constitutively active STAT5 also rescued IL-9 expression and STAT5 binding to the *Ii9* promoter was confirmed. In a papain-induced model of lung inflammation, it was shown that in the absence of Itk, cardinal symptoms of asthma, such as AHR, mucus production and eosinophilia, were markedly reduced compared to wild-type mice. Furthermore, less Th2, Th9 and IL-9-producing ILC2 cells were found in the lungs of Itk^{-/-} mice, while intriguingly, in the early phase of allergen challenge, IL-9⁺ ILC2 cells were not diminished. The authors attributed this latter effect to a lack of IL-2 secretion from Itk-deficient T cells [72].

Factors inhibiting IL-9 production

Besides many factors that positively regulate Th9 differentiation, there are also inhibitory factors (Table 1). Recently, it has been demonstrated that the cyclooxygenase-2 (COX-2)-derived prostaglandins PGE2 and PGD2 are important in the control of Th9 cell development in vitro and in vivo. Mice lacking COX-2 and sensitized and challenged with OVA

displayed increased numbers of Th9 cells in the blood, lung, BALF and lung draining lymph nodes. Furthermore, the group showed that the absence of endogenous COX-2 in naïve CD4⁺ T cells amplifies their expression of IL-9 and other Th9 lineage markers when polarized under Th9-favouring conditions in vitro. The authors also demonstrated that the two prostaglandins PGE2 and PGD2 were able to suppress Th9 cell development in vitro and in vivo by suppressing the expression of the IL-17RB. Finally, they showed that PGE2 and PGD2 suppressed Th9 development from naïve human T cells in vitro [81].

Tyrosine kinase 2 (Tyk2) is known to relay signaling of different cytokines and IFNs, such as IL-6, IL-10 or IFNα. Furthermore, Tyk2 has been shown to have a protective role in asthma, as Tyk2-deficient mice display hypereosinophilia and high serum IgE levels in a murine model of the disease. Moreover, in the absence of Tyk2, Th2 and Th9 responses are increased, while Th17 cell differentiation was observed to be diminished. The finding that both *Irf4* and *Spi1* mRNA expression was enhanced in total lung cells of Tyk2^{-/-} asthmatic mice is in accordance with the discovery of heightened IL-9 secretion from lung CD4⁺ T cells in these mice, suggesting a regulatory role of Tyk2 in Th9 differentiation [73].

As in Tyk2 deficiency, also lack of the E3 ubiquitin ligase Cbl-b has been shown to increase Th2 and Th9 cells in an adoptive transfer model of allergic asthma, accompanied by, e.g. lung eosinophilia and increased airway resistance. Mechanistically, in this study, it has been demonstrated that STAT6 is able to bind Cbl-b after IL-4 stimulation, intensified by TCR/CD28 stimulation. Thus, ubiquitination of STAT6 by Cbl-b was revealed as a regulatory process in Th9 differentiation [79].

By using a chronic *Aspergillus fumigatus*-induced model of airway disease, Kerzerho et al. demonstrated that in the absence of the co-stimulatory molecule programmed cell death ligand 2 (PD-L2), the number of Th9 cells in the lung was increased as compared to wild-type control animals. Additionally, it was observed that this was accompanied by higher TGFβ levels in whole lung lysates from PD-L2-deficient mice. Moreover, the researchers found that PD-L2 neutralization and deficiency enabled in vitro generated Th9 cells to produce more IL-9 than PD-L2 competent cells. Together the findings indicate an inhibitory role of PD-L2 on Th9 cell differentiation [26].

Vitamin D has been described to have immunosuppressive effects and a lack of vitamin D is thought to be unfavourable in asthma. Analysis of PBMCs from asthma patients and healthy controls revealed that in vitro culture with both 1,25(OH)₂cholecalciferol and 25(OH)cholecalciferol reduced IL-9 production in memory CD4⁺ T cells. Adding the steroid dexamethasone to the culture further strengthened this effect. These data indicate that vitamin D, especially in the clinically relevant combination with steroids, could be explored as a therapeutic agent in asthma to inhibit Th9 responses [84].

Moreover, different metabolic pathways have also been linked to the differentiation and function of T cells. Recently, it was observed, that the histone deacetylase SIRT1 is involved in IL-9 expression in Th9 cells. SIRT1 has been described to modulate stress responses and energy metabolism as well as inflammatory processes. While in allergic airway disease SIRT1 was shown to drive Th2 cell differentiation, it was now reported to be a negative regulator of IL-9 expression, as a T cell-specific deficiency of SIRT1 led to enhanced IL-9-producing T cells, accompanied by airway inflammation and eosinophilia, which was reversed by anti-IL-9 antibody treatment [83].

IL-9-mediated effects on target cells in allergic asthma

IL-9 has biological effects on different target cells that express the IL-9R on their surface. Many of these target cells are involved in asthma pathogenesis, as shown in Fig. 2. The IL-9R consists of two subunits, the α -chain (IL-9R α) and the common γ -chain receptor shared by IL-2, IL-4, IL-7, IL-15 and IL-21. Binding of IL-9 to its receptor leads to cross-phosphorylation of Janus kinase 1 (JAK1) and JAK3. This then leads to the activation of STAT1-STAT5 homodimers or STAT1-STAT3 heterodimers [87–89].

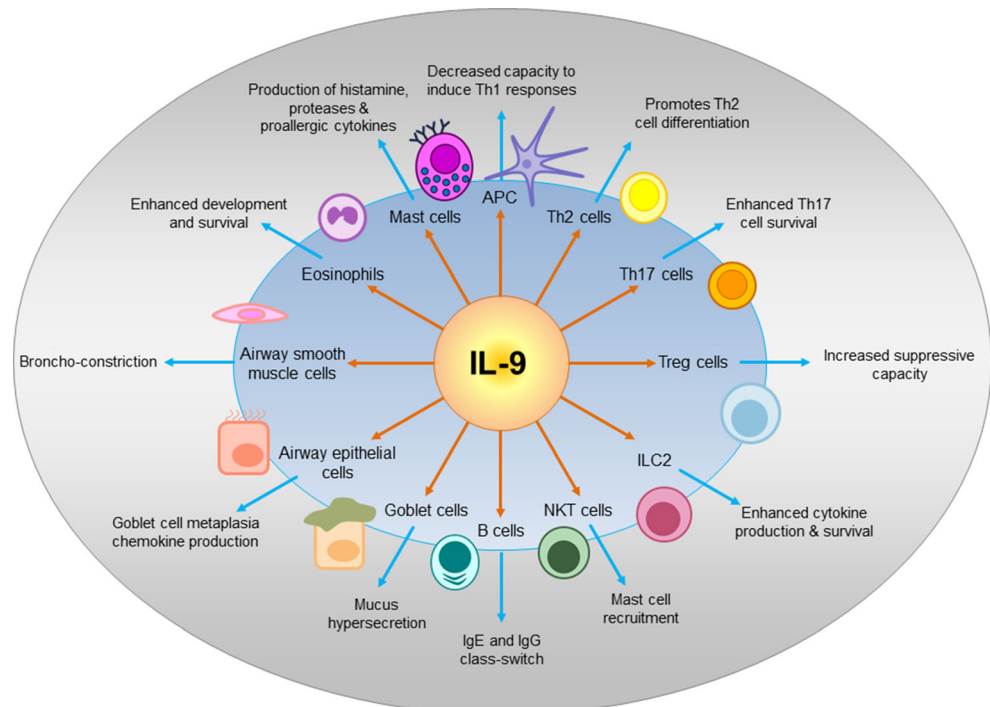
Cells of the immune system

It has been reported that IL-9 is able to target professional antigen-presenting cells (APCs) and to limit their capacity to

induce Th1-type immune responses. IL-9 promotes TGF β expression in a heterogeneous population of macrophages and monocytes that is activated through lipopolysaccharide (LPS). This led to a decrease in the oxidative burst and TNF expression of these cells [90]. Furthermore, a culture of PBMCs, which include T cells and monocytes, with IL-9 decreased the production of IFN γ and IL-12 in response to *Mycobacterium tuberculosis* extract. Additionally, blocking IL-9 in these cultures induced the production of IL-12 in these PBMCs [91].

Mast cells are a main target of IL-9 which serves as a growth factor for these cells and also influences their function. In combination with stem cell factor (SCF), IL-9 promotes the growth of bone marrow mast cells and mast cell progenitors. Additionally, it is known that IL-9 alone or together with SCF or in combination with Fc ϵ RI activation can promote the expression of proteases and pro-allergic cytokines including IL-1 β , IL-5, IL-6, IL-10 and IL-13 in cultured mast cells [22, 92–95]. Especially, the cytokines IL-5 and IL-13 are of interest because they are central mediators of allergic asthma. IL-13 and IL-5 production promotes the survival and migration of eosinophils. Furthermore, IL-13 supports the activation of macrophages, increased the permeability of and mucus production by airway epithelial cells and has an impact in collagen deposition and AHR [96]. Therefore, IL-9 also has indirect effects in the airway. Studies also revealed that the accumulation of mast cells during an acute allergic inflammation model is dependent on IL-9, because neutralizing IL-9 dramatically reduced the number of lung-infiltrating mast cells. Additionally, treatment with anti-IL-9 antibodies led to reduced mast cell protease expression [30].

Fig. 2 Effects of IL-9 on target cells relevant in allergic asthma. IL-9 (centre circle) has an impact on many different cell types (middle circle). The influence on target cells in asthma is shown in the outer circle



The IL-9R seems to be expressed also on eosinophils from human peripheral blood. Stimulation of these eosinophils with IL-9 upregulated the cell surface expression of the IL-5R α chain and increased their survival. Additionally, it has been demonstrated that IL-9 increased eosinophil development from CD34⁺ progenitors by mechanisms that include the up-regulation of the IL-5R α chain. Therefore, IL-9 may potentiate the in vivo eosinophil function by increasing their survival and IL-5-mediated differentiation and maturation [97].

ILC2 cells are known to constitutively express the IL-9R on their surface which suggested an autocrine effect of IL-9 on ILC2 cell activation and cytokine production. Mohapatra et al. could demonstrate that IL-9 deficiency in ILC2 cells leads to a decrease in IL-5 and IL-13 production when cultured in the presence of TSLP and IL-33, while the number of ILC2 cells was not affected [98].

Beside its effect on cells of the innate immune system, also adaptive immune cells are influenced by IL-9. In B cells, IL-9 seems to influence their development as well as their function. It facilitates IL-4-mediated production of IgE and IgG from human as well as murine B cells without affecting IgM production [99, 100]. Furthermore, increased IgE was paralleled by an upregulation of IL-9R α and CD27, with the latter being a memory B cell marker implicated in increased IgE secretion. Thus, IL-9 seems to be involved in memory B cell differentiation [101].

Referring to the different Th subsets, IL-9 has been shown to act as a growth factor for these cells, especially for Th17 cells. Previous studies demonstrated that IL-9 is able to promote the expansion of Th17 cell populations in vitro. In contrast, IL-9 does not seem to facilitate the expansion or survival of FoxP3⁺ Treg cells but enhances their regulatory function in vitro [20]. Furthermore, pulmonary overexpression of IL-9 seems to induce Th2 cytokine expression, namely IL-5 and IL-13, resulting in pathologic changes in the lungs [29].

Structural cells in the lung

Airway smooth muscle cells are known to express the IL-9R. Therefore, IL-9 can directly act on these cells by promoting the release of the chemokines CCL11 and IL-8 to be attractants for eosinophils and neutrophils respectively [102, 103]. The IL-9-mediated expression of CCL11 is STAT3 dependent which directly binds to the *ccl11* promoter [104].

IL-9 can also directly affect human primary airway epithelial cells and cell lines by inducing the expression of mucins, e.g. MUC4, thereby inducing mucus cell metaplasia. Furthermore, IL-9 also induces the expression of chemoattractant factors from human airway epithelial cells in a dose-dependent manner, such as IL-16 and regulated on activation, normal T cells expressed and secreted (RANTES), which are chemotactic for T cells [105]. Additionally, IL-9 promotes the expression of CC-type chemokines in murine primary lung epithelial cells and cell lines that are chemotactic for eosinophils [106].

Concluding remarks

Allergic asthma still is a health burden, especially in Western countries, and the importance of IL-9-producing cells has been observed by many different groups. IL-9 is produced by CD4⁺ T cells as well as other cells such as mast cells, innate cells and eosinophils. It is known to promote the proliferation of activated T cells, increase IgE production of B cells and promote the differentiation and proliferation of mast cells. Therefore, IL-9 is linked to the pathogenesis of asthma and studies in mice and humans demonstrated a link between IL-9 production and, e.g. AHR, lung eosinophilia, mast cell accumulation and mucus hypersecretion. As the signaling pathways of Th9 and other IL-9-producing cells are further elucidated, these data encourage the use of IL-9 blockade as a therapeutic strategy in asthma patients. Even though the direct inhibition of IL-9 by administration of anti-IL-9 antibodies so far did not revolutionize asthma therapy, there are promising approaches to pursue this goal, e.g. with the tick-derived saliva protein sialostatin L. Taken together, the contributing role of IL-9-producing cells to asthma has been elucidated and opens many possibilities to take advantage for the development of novel therapeutic strategies, but so far, these still need further exploration.

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