The role of T-cell co-stimulatory molecules in murine models of allergic asthma

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Cover design: Martijn Pieck (Dienst Grafische Vormgeving en Fotografie, Faculty of Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands)

Financial support by:

GlaxoWellcome

Greiner Bio-one

the Netherlands Asthma Foundation

the Stichting AstmaBestrijding

for the publication of this thesis is gratefully acknowledged.

Printed by: PrintPartners Ipskamp, Enschede, The Netherlands

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De rol van T-cel co-stimulatie moleculen in
muizenmodellen voor allergisch astma
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties,
in het openbaar te verdedigen op
woensdag 30 oktober 2002 des middags te 12.45 uur

door
Daphne Thérèse Deurloo
geboren op 18 januari 1974 te Zwolle

Promotor: Prof. dr. Frans P. Nijkamp
Co-promoter: Dr. Antoon J.M. van Oosterhout

The studies presented in this thesis were performed at the Department of Pharmacology and Pathophysiology, Faculty of Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

The Netherlands Asthma Foundation is gratefully acknowledged for the financial support of the studies presented in this thesis (Research Grant 3.2.97.09)

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Chapter 1

General introduction

1 Allergic asthma

Allergic asthma is a chronic inflammatory disorder of the airways characterized by reversible airflow obstruction, persistent airway hyperresponsiveness, chronic airway inflammation and airway remodeling (1). Airway hyperresponsiveness is an increased sensitivity of the airway smooth muscle to a wide variety of bronchospasmogenic stimuli such as methacholine. As a result of the chronic inflammation, airway tissue is continuously being injured and healed resulting in structural changes (called remodeling) of airway tissue that may account for the decline in airway function seen in asthmatic patients over several years (2).

The term allergy was introduced in 1906 by Clemens from Pirquet as "an altered capacity of the body to react to a foreign substance" (3), which was an extremely broad definition that included all immunological reactions. Allergy is now defined as a symptomatic reaction to an innocuous environmental antigen (allergen). It results from the interaction between the antigen and immunoglobulin(Ig)E antibodies or primed T-cells produced by earlier exposure to the same antigen (sensitization) (4).

The etiology of asthma is complex and multifactorial, involving the interaction between genetic and environmental factors. The term atopy from the Greek *atopos*, meaning "out of place" is used for the state of individuals that have an exaggerated tendency to produce IgE antibodies against allergens and have clinical manifestations of one, or more, atopic diseases (*i.e.* allergic rhinitis, asthma and atopic eczema) (4). Some allergic diseases operate (*e.g.* contact dermatitis and hypersensitivity pneumonitis) operate through IgE-independent mechanisms and in this sense can be considered as non-atopic, allergic conditions. Also a subgroup of asthmatic patients demonstrates no signs of atopy (5). These asthmatics show negative skin tests and there is no clinical or family history of allergy. Furthermore, serum total IgE concentrations are within the normal range and there is no evidence of specific antibodies directed against common allergens.

Several genes or gene families are involved in asthma and allergy: genes that are inflammation-specific, sensitization-specific and genes specific for the type of asthma or allergic disease (6).

The incidence, morbidity and mortality of asthma have increased worldwide over the last two to three decades (7). This observation is best explained by changes in environmental factors, such as early childhood infection, environmental pollution, allergen exposure, and diet and changes in other factors including increased awareness of the disease, improved diagnostics and psycho-social influences (8-10).

1.1 The allergic inflammatory response

Professional antigen-presenting cells (APCs), such as dendritic cells (DCs), macrophages and B-cells, initiate immune responses to inhalant antigens that have

penetrated the respiratory mucosa. In animal models of allergic asthma, the DCs have been identified as the principal APC involved in the induction and maintenance of Th2 allergic responses (11). Upon antigen up-take, DCs traffic to T-cell rich regions of lymphoid tissue and display processed peptide fragments of the antigen in the context of major histocompatibility complex (MHC) class II molecules to naïve antigen-specific CD4+ (helper) T-cells (Figure 1).

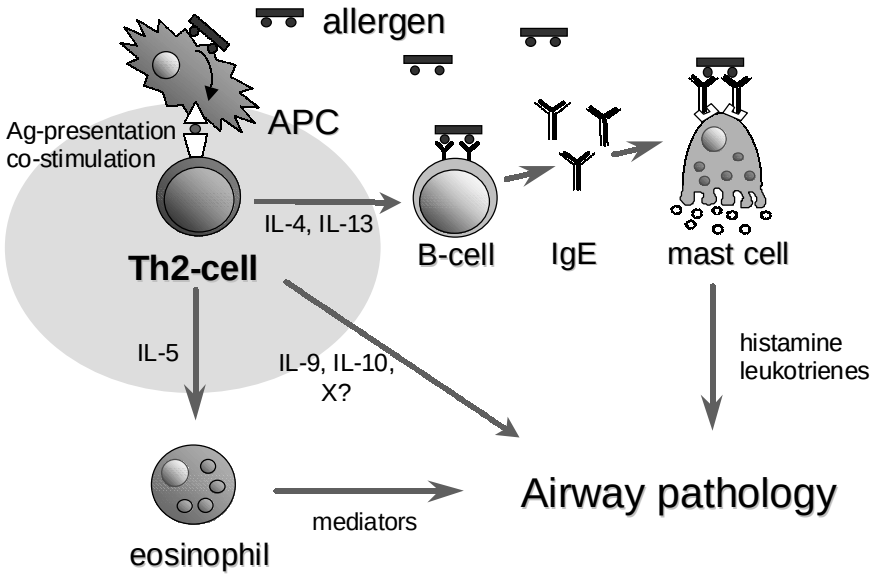

Figure 1:Schematic representation of the pathophysiology of allergic asthma.

Clonal expansion is the proliferation of antigen-specific lymphocytes in response to antigenic stimulation and precedes their differentiation into effector cells. Experiments in mice have shown that there can be a substantial increase (~ 1000-fold) in the precursor frequency of antigen-specific T-cells in immune animals compared with naïve animals (*see* 12).

Second, activated T helper cells differentiate into one of the two distinct subsets on the basis of their cytokine profile. This classification was first proposed for mouse T-cells by Mosmann *et al*. (13) and subsequently for human T-cells by Kapsenberg *et al.* (14). CD4+ T helper 1 (Th1) cells are characterized by the production of interleukin-2 (IL-2) and interferon-γ (IFN-γ), whereas Th2 cells produce IL-4, IL-5, IL-9, IL-10 and IL-13. Th2 cells are optimized to combat helminithic parasite infections. However, Th2 cells are also the dominant Th phenotype observed in patients with allergic disorders such as asthma (15-17). Many subsequent studies in mice and man have confirmed the pivotal role of allergen-specific Th2 cells in the

pathogenesis and maintenance of allergic asthma, their cytokines orchestrating the inflammatory immune response resulting in airway symptoms (reviewed in 18).

IL-4 favors the development of Th2 cells, thus perpetuating the Th2 response (19, 20). Furthermore, IL-4 and IL-13 are required for the induction of proliferation and isotype switching to IgE by B-cells that recognize the allergen (21, 22). Antigen-specific IgE binds to the high-affinity receptor for IgE, FcεRI (CD64) on mast cells (sensitization). Upon re-exposure to the same allergen (challenge), submucosal mast cells in the lower airways will be activated when their surface-bound IgE is cross-linked by the allergen, resulting in the release of large amounts of inflammatory mediators, including histamine and leukotrienes (23). These short-lived mediators are responsible for the early asthmatic phase reaction that occurs within seconds and consists of bronchoconstriction and increased secretion of fluid and mucus, making breathing more difficult by trapping inhaled air in the lungs. The immediate allergic reaction usually resolves within one to two hours, but is followed by a more excessive inflammation, known as the late-phase response. This late phase involves leukotrienes, cytokines and chemokines, which recruit and activate other effector cells, notably Th2 lymphocytes, eosinophils and basophils that induce airflow obstruction, mucus secretion, smooth muscle hypertrophy, epithelial shedding and thickening of the bronchial wall (24, 25).

The Th2 cytokine IL-5 is an important mediator for the development, migration, and activation of eosinophils. This granulocyte is always present in the allergic lung, but its precise role in the pathogenesis of allergic asthma or more specifically its role in the late-phase response or development in non-specific airway hyperresponsiveness is still a matter of debate (26, 27).

An important feature of asthma is chronic inflammation of the airways, which is characterized by the continued presence of increased numbers of Th2 lymphocytes, eosinophils and other leukocytes (24). Although allergic asthma is initially driven by a response to a specific allergen, the subsequent chronic inflammation seems to be perpetuated even in the apparent absence of further exposure to allergen, resulting in airway hyperresponsiveness to non-specific stimuli and airway remodeling. At present, treatment of asthma consists of long-term suppression of airway inflammation plus relief of symptoms with quick-acting bronchodilators (primarily dry powder β_2-adrenoceptor agonists). Inhaled corticosteroids are the most effective agents available for the reduction of inflammation and improvement of pulmonary function, but their potential side effects when used in escalating doses have led to adjunctive therapies including long-acting β_2-agonists, sustained-release theophylline and leukotriene antagonists (25, 28). In view of the crucial role of allergen-specific Th2 cells in allergic asthma, these cells might be an interesting and more specific therapeutic target (29, 30).

2 T-cell co-stimulation

The current concept of how lymphocytes become activated is originally derived from the two-signal hypothesis from studies with B-cells first proposed by Bretscher and Cohn in 1970 (31) and subsequently expanded to T-cells by Lafferty and Cunningham (32). After modifications by others, it is generally believed that optimal activation of lymphocytes requires specific antigen recognition by the T-cell receptor (TCR), presented in the form of processed peptides in association with MHC class II molecules (signal 1) and additional signals (collectively called signal 2 or co-stimulatory signals), delivered by the same APC (33, 34). APCs are activated to express co-stimulatory ligands upon binding and ingesting pathogens through receptors of the innate immune system.

This requirement for the simultaneous delivery of antigen-specific and co-stimulatory signals by one cell in the activation of T-cells means that only such activated APCs can initiate T-cell responses and prevents uncontrolled activation of potentially self-reactive by broadly expressed self-antigens in non-lymphoid tissues, so-called peripheral self-tolerance. Thus, the two-signal theory is one of the major mechanisms limiting the development of destructive immune responses to self-tissues (auto-immunity). Indeed, antigen binding to the TCR in the absence of co-stimulation not only fails to activate the cell; it also leads to a state of antigen-specific unresponsiveness, called anergy. Such anergic T-cells are refractory to antigen-specific re-stimulation even when the antigen is presented by a professional APC (35-39).

2.1.1 The CD28/CTLA4:B7-1/B7-2 pathway

Although many receptor-ligand pairs of T-cell co-stimulation have been identified (40), the CD28/CTLA4:B7-1/B7-2 pathway is the primary pathway of T-cell co-stimulation (reviewed in 41-44) (Figure 2 on page 13).

CD28 is member of the Ig superfamily with a single Ig variable-like extracellular domain in addition to its transmembrane and cytoplasmic domains. Expressed as a disulphide-linked homodimeric glycoprotein, it is constitutively present on the surface of naïve T-cells. Following T-cell activation, the levels of CD28 increase (45).

The ligands of CD28, B7-1 (CD80) and B7-2 (CD86), are also members of the Ig superfamily, with two Ig-like extracellular domains (one variable-like and one constant-like). On most APC populations, B7-2 is expressed constitutively at low levels and is rapidly up-regulated, whereas B7-1 is inducibly expressed later after activation. The earlier expression pattern of B7-2 suggests that B7-2 is the more important co-stimulator for initiating T-cell responses. However, this remains to be substantiated. B7-1 and B7-2 appear to support T-cell activation equivalently (46). Differential outcomes of the CD28:B7-1 versus CD28:B7-2 interaction seem mainly The ligands of CD28, B7-1 (CD80) and B7-2 (CD86), are also members of the Ig

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CD28 signals promote T-cell activation by augmenting and sustaining T-cell responses initiated by TCR signaling. CD28 co-stimulation has been shown to stimulate proliferation by increasing the secretion of the autocrine growth factor IL-2 and the induction of IL-2 receptor expression. In addition, CD28 increases T-cell survival by the induction of the anti-apoptotic factor Bcl-$x_{L.}$ Some reports indicate that CD28 ligation might favor differentiation of T-cells into Th2 cells (47-52). Furthermore, CD28 plays an important role in B-cell differentiation and germinal center formation (53). Finally, CD28 also directs T-cell migration into inflammatory sites by inducing the production of specific chemokines and the regulation of chemokine receptors (54). In the absence of TCR signaling, CD28 engagement does not usually have a physiological effect.

The second receptor for the B7 ligands, cytotoxic T-lymphocyte antigen 4 (CTLA4; CD152), is expressed on T-cells only after a period of activation and delivers inhibitory signals. Cell surface expression of CTLA4 is very tightly regulated, with most of the CTLA4 protein residing within cytoplasmic vesicles (55). CTLA4 shares about 30% identity with CD28 at the amino acid level and is also an Ig superfamily protein with a single extracellular variable-like domain expressed as a disulphide-linked homodimer that is capable of binding the B7 molecules, but with a much higher affinity (56). This suggests that CTLA4, as it becomes expressed on the surface of activated T-cells, might preferentially interact with B7-1 and B7-2, and thus aid in the termination of immune responses.

CTLA4 inhibits T-cell proliferation by reducing IL-2 production and IL-2 receptor expression and by arresting T-cells at the G1 phase of the cell cycle (57-59). In addition, CTLA4 has been shown to limit the extent of Th2 differentiation (60-63). The importance of negative regulatory signals through CTLA4 is illustrated in mice deficient in CTLA4 ($CTLA4^{-/-}$). These mice die within three to four weeks after birth expansion from a lymphoproliferative disease characterized by CD28-mediated polyclonal T-cell activation and progressive infiltration of T-cells in critical visceral organs (64-66). Recent studies also indicate that CTLA4 not only serves to counterbalance CD28 signals but also can inhibit immune responses independently of CD28 (67-69).

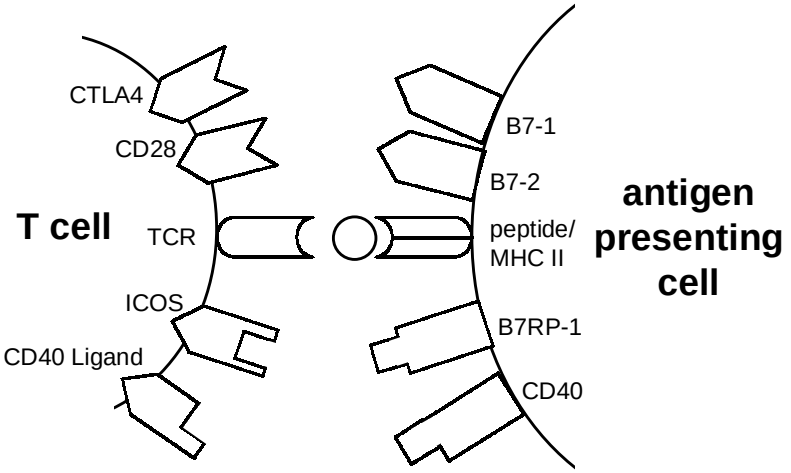

Figure 2:Schematic representation of some T-cell co-stimulatory receptors and their ligands.

Because CTLA4 has a higher affinity for the B7 ligands compared with CD28, CTLA4-Ig has been widely used to block T-cell co-stimulation in an array of experimental animal models of T-cell-mediated diseases (41, 56).

CTLA4 engagement has also been implicated in the induction of peripheral tolerance (60, 70-72), which might be induced not because of the absence of B7-1/ B7-2-mediated co-stimulation but as a consequence of CTLA4:B7-1/B7-2 interactions.

2.1.2 The ICOS:B7RP-1 pathway

Although CD28 is a key co-stimulatory molecule, it does not account for all co-stimulatory function in T-cells. Studies *in vitro* have demonstrated that effector and memory T-cells are less dependent on co-stimulation in comparison with naïve T-cells (48, 73, 74), whereas studies *in vivo* on the effectiveness of giving CTLA4-Ig after priming of immune responses have yielded conflicting results (75). These results suggested that alternative co-stimulatory pathways may exist.

One of the alternative T-cell co-stimulatory molecules is the recently discovered, third member of the CD28 family member inducible co-stimulator (ICOS) (reviewed in 44, 76, 77) that has been cloned in human (78, 79), mice (80, 81) and rat (82). ICOS shares approximately 30-40% sequence similarity with CD28 and CTLA4 and also possesses a single extracellular Ig-variable-like domain. However, despite its structural similarities to CD28 and CTLA4, ICOS does not bind to B7-1 and B7-2 but binds to its own ligand B7 related protein 1 (B7RP-1), a novel B7 family

member (83, 84) (also referred to as B7h (85), GL50 (86), or LICOS (87) in mice and as B7-H2 (88) or ICOSL (89) in humans).

ICOS is rapidly expressed on the surface of T-cells as a disulphide-linked homodimer after TCR engagement and is retained on many memory T-cells (78, 80, 81, 83, 90, 91), suggesting that ICOS provides co-stimulatory signals to activated T-cells. Indeed, during the initial activation of naïve T-cells, ICOS signals only modestly influence T-cell proliferation and IL-2 production in comparison with CD28 signaling (78, 80, 81, 83, 88, 91). However, ICOS co-stimulation is equivalent to that mediated by CD28 for the production of Th effector cytokines IL-4, IL-5, IFN-γ, and TNF-α and is more effective in enhancing IL-10 production (78, 84, 88, 90-95). $ICOS^{-/-}$ mice demonstrated that ICOS has a critical role in Ig isotype class switching and germinal center formation (96-98).

Compared with B7-1 and B7-2, B7RP-1 has a unique expression pattern, in that it is differentially induced on lymphoid tissues and is also expressed in non-lymphoid tissues in mice (85-87) and humans (87, 99). The up-regulated expression of B7RP-1 in peripheral tissues by inflammatory cytokines might indicate a physiological role of B7RP-1 in the reactivation of memory and effector T-cells (85, 99).

2.1.3 Other members of the CD28 and B7 families

Since the unraveling of the human genome, the CD28 and B7 families are rapidly growing (reviewed in 44, 77, 100, 101) and now also count programmed death (PD)-1 (102) and PDL-1 (B7-H1) (103) and PD-L2 (B7-DC) (104, 105) as members, respectively. Interaction of PD-1 with either PD-L1 or PD-L2 has been shown to result in inhibition of T- and B-cell responses (104, 106). Interestingly, PD-L1 and PD-L2 have also been reported to co-stimulate T-cell responses (103, 105) raising the possibility that there may be additional receptor(s) for these ligands, in analogy with the B7-1/B7-2:CD28/CTLA4 system.

The newest member of the B7 family, B7-H3 appears to engage a co-stimulatory molecule on activated T-cells that does not correspond to any of the known CD28 family members (107). B7-H3-Ig fusion protein co-stimulates CD4+ and CD8+ T-cell proliferation, IFN-γ production and CD8+ lytic activity.

Moreover, generation of mice lacking both CD28 and CTLA-4 has produced evidence for a third B7-1/B7-2 receptor (108).

2.2 Receptor-ligand pairs involved in T-cell co-stimulation from the TNF families

Receptor-ligand pairs involved in T-cell co-stimulation are also found in the rapidly expanding tumor necrosis factor (TNF) receptor-ligand superfamilies (reviewed in 109-111). A common function of these receptor-ligand pairs is the regulation of cell-mediated activation and/or apoptosis, in other words, the regulation of life and

death. Molecules with T-cell co-stimulatory function and their ligands include CD27:CD27L (CD70), CD40:CD40L (CD154), OX40(CD134):OX40L and 4-1BB:4-1BBL (*see* Table 1).

2.2.1 The CD40L:CD40 pathway

The best-characterized pair of counter-receptors from this family is CD40 ligand (CD40L; CD154) transiently expressed on activated CD4+ T-cells that interacts with CD40 found on many cell types, including APCs (reviewed in 112, 113). In addition to evidence for direct T-cell co-stimulation *via* CD40L (114-119), triggering of CD40 contributes indirectly to T-cell co-stimulation by the up-regulation of the B7-1 and B7-2 expression on APCs, thus enhancing T-cell activation through CD28-mediated signaling (120, 121). Ligation of CD40 on B-cells, together with IL-4 (21), is necessary for their activation and the induction of isotype switching to IgE (122).

2.3 Yet other receptor-ligand pairs involved in T-cell co-stimulation

Some other pathways that have been shown to co-stimulate T-cells are listed in Table 1.

Table 1: Overview of T-cell co-stimulatory receptors and their ligands. Adapted from (40, 101)

Receptor on T-cell	Ligand(s)
CD28 and B7 subfamilies of the Ig superfamily	
CD28	B7-1 (CD80)/B7-2 (CD86)
CTLA4 (CD152)	B7-1 (CD80)/B7-2 (CD86)
ICOS	B7RP-1 (B7h, GL-50, LICOS, B7-H2, ICOSL)
PD-1	PD-L1 (B7-H1)/PD-L2 (B7-DC)
unknown yet	B7-H3 (B7RP-2)
Tumor necrosis factor receptor/ligand superfamilies	
CD40L (CD154)	CD40
OX40 (CD134)	OX40L
4-1BB (CD137)	4-1BBL
CD27	CD27L (CD70)
LIGHT	HVEM
Other receptor-ligand pairs of T-cell co-stimulation	
CD2	LFA-3 (human CD58; mouse CD48)
LFA-1 (CD11a:CD18)	ICAM-1 (CD54)/ICAM-2 (CD102)
ICAM-3	DC-SIGN (CD209)
unknown yet	heat-stable antigen (HSA; CD24)
dipeptidyl peptidase IV (CD26)	adenosine deaminase
CD81	unknown yet
OX-2 (CD200)	unknown yet
CD100	plexin-B1 (non-lymphoid tissues) CD72 (lymphoid tissues)
SLAM (CD150)	measles virus, CD46 and SLAM itself

3 Murine models of allergic asthma

To study the cellular and molecular mechanisms involved in the pathogenesis of allergic asthma, murine models have been very useful (123, 124). Many pathogenic mechanisms such as the central role of Th2 cells were first elucidated in mice and subsequently confirmed in humans. In general, mice are injected parenterally with a model antigen like ovalbumin (OVA), eggs and antigen of the *Schistosoma mansoni* parasite (125) or human relevant antigens (*e.g.* derived from house dust mite (126), the fungus *Aspergillus fumigatus* (127) or cockroach (128)) to induce systemic sensitization. This sensitization usually occurs in the presence of the adjuvant aluminum hydroxide that induces a Th2-dominant response *in vivo*. After sensitization, antigen-specific IgE can be detected in the sera of mice. Subsequently, the same antigen is administered once or repeatedly through the airways to focus the inflammatory process in the bronchi and lungs. Upon antigen challenge, several characteristic features of the clinical disease can be observed in mice. These features include airway hyperresponsiveness *in vivo* to bronchospasmogenic stimuli such as methacholine, an influx of inflammatory cells in the broncho-alveolar lavage fluid (BALF) and lung tissue, mucus production and up-regulated levels of antigen-specific IgE in serum (129). Mice can develop both an early and late-phase "asthmatic" responses (130). Murine models can also be used to study various aspects of airway remodeling after protocols involving chronic challenge with antigens (128, 131). Because the mouse is broadly used as experimental animal, many transgenic and gene deficient strains have been generated (132) and diverse mouse-specific antibodies and recombinant proteins (*e.g*. cytokines) are available for experimental interference.

However, the extrapolation from these models to the human condition is not straightforward (133, 134), as reflected by some recent clinical disappointments; anti-IL-5 mAb (135) and IL-12 treatment (136) did not seem to modulate the late asthmatic response or airway hyperresponsiveness. These studies relied on a "single" mediator hypothesis, and strategies designed to suppress inflammatory cell function might prove more successful. In addition, in most studies, experimental treatment is administered during the development of asthma manifestations in mice, whereas patients start therapy after the development of airway symptoms. In general, continued emphasis on how the disease process can be best modeled is warranted to optimize murine models as a tool for testing new forms of therapies (133, 134, 137).

3.1 The role of T-cell co-stimulation in murine models of allergic asthma

Also the role of various T-cell co-stimulatory molecules has been studied in murine models of allergic asthma.

3.1.1 The CD28/CTLA4:B7-1/B7-2 pathway

The critical role of CD28 signaling in the development of Th2-dominated allergic inflammatory responses in mice has been demonstrated in both gene targeting and blocking studies. No asthma-like symptoms could be induced in $CD28^{-/-}$ mice (51, 52, 138) or B7-1/B7-$2^{-/-}$ mice (139). Administration of CTLA4-Ig or monclonal antibodies (mAbs) against the B7 molecules at the time of antigen sensitization or challenge of wild-type mice (140-144) greatly reduced the allergen-induced manifestations of asthma.

3.1.2 The ICOS:B7RP-1 pathway

$ICOS^{-/-}$ mice were susceptible to the influx of inflammatory cells in the BALF induced by airway challenge in primed mice (96). In blocking studies, the ICOS:B7RP-1 pathway was interrupted during antigen-sensitization and/or airway challenge (80, 125) or solely during the final aerosol challenge (92). Collectively, these studies suggest that ICOS co-stimulation may contribute more to the effector phase than to the initiation of Th2-dominated allergic inflammatory responses (80, 92, 96, 125).

3.1.3 The CD40L:CD40 pathway

The importance of the CD40L:CD40 pathway in the induction of asthma-like symptoms has only been investigated in mice deficient in either CD40L or CD40 (145-147). Because ligation of CD40 on B-cells, together with IL-4 (21), is required for their activation and IgE isotype switching (122), the absence of (allergen-specific) IgE in serum was anticipated in all of these studies. However, conflicting results were obtained regarding the involvement of the CD40L:CD40 pathway in the development of airway hyperresponsiveness and BALF eosinophilia.

3.1.4 Other receptor-ligand pairs involved in T-cell co-stimulation

Manifestations of allergic asthma were reduced in $OX\text{-}40^{-/-}$ mice (148), presumably because of its regulation of the number of CD4+ T-cells that persist over time (149).

In OVA-sensitized and challenged $CD81^{-/-}$ mice, similar levels of OVA-specific IgE were found, whereas airway hyperresponsiveness and lung inflammation were reduced compared with wild-type mice (150). In immunized $CD81^{-/-}$ mice, antigen-specific cytokine production by the draining lymph nodes was dramatically reduced but proliferation was unaffected, as was cytokine production upon polyclonal stimulation. Moreover, a slight increase in T-cells and a considerable decrease in B-cells in peripheral lymphoid tissues were observed. Perhaps, the impaired Th2 response *in vivo* is due to the deficiency in T-cell interactions with B-cells and other APCs (150).

4 Blockade of T-cell co-stimulation in humans

Administration of CTLA4-Ig to patients with *psoriasis vulgaris* in a phase I trial produced a dose-dependent improvement in skin lesions (151, 152). Also in patients with *rheumatoid arthritis*, CTLA4-Ig and a second-generation molecule based on CTLA4-Ig (LEA29Y) produced improvements in the signs and symptoms of the disease according to standard criteria (153). After administration of recombinant CD40L to cancer patients, encouraging antitumor activity, including a long-term complete remission, was observed (154).

5 Aim and outline of this thesis

In this thesis, the role of various T-cell co-stimulatory molecules in OVA-induced models of allergic asthma in BALB/c mice was further investigated to determine if blockade of T-cell co-stimulation might be of therapeutic interest.

A recent study using a novel mouse strain lacking both CD28 and CTLA4 demonstrated considerable residual CD4+ T-cell proliferation *in vitro* that was dependent on B7-1 or B7-2, suggesting the existence of an additional, third B7-1/B7-2 receptor (108). In addition, CD28/CTLA4$^{-/-}$ mice mounted a substantial Th1-mediated cardiac allograft rejection response *in vivo*, in contrast with long-term allograft survival in B7-1/B7-2$^{-/-}$ mice (155, 156). In the study described in **chapter 2**, it was investigated if T-cell co-stimulation *via* the putative B7-1/B7-2 receptor contributes to the induction of Th2-mediated manifestations of allergic asthma using this novel CD28/CTLA4$^{-/-}$ mouse strain.

In contrast to animal studies in which the effect of an experimental treatment on the development of asthma-like symptoms is examined, asthma patients usually start therapy after the development of airway symptoms, while exposure to environmental allergen mostly continues. Studies *in vitro* have demonstrated that effector and memory T-cells are less dependent on co-stimulation in comparison with naïve T-cells (48, 73, 74), whereas studies *in vivo* on the effectiveness of giving CTLA4-Ig after priming of immune responses have yielded conflicting results (75). To more closely resemble the OVA-induced animal model of allergic asthma to the clinical situation, we designed a new protocol in which treatment starts after development of airway inflammation while OVA exposures are continued (ongoing model). After validation with the "gold-standard" corticosteroid dexamethasone, CTLA4-IgG was applied in this ongoing murine model of allergic asthma (**chapter 3**).

Studies on the role of ICOS in murine models of allergic asthma have suggested that ICOS has a more prominent role in the effector phase than in the initiation of Th2-dominated allergic inflammatory responses (80, 92, 96, 125). Therefore, the effects of ICOS blockade during the development of asthma-like symptoms in mice (induction model) were compared with the effects of ICOS blockade after the

development of airway symptoms when OVA exposures were continued (ongoing model) (**chapter 4**).

In the absence of co-stimulation, TCR triggering results in the development of antigen-specific T-cell anergy that is unresponsiveness to antigenic re-stimulation in the presence of co-stimulation (Refs. 35-39 and paragraph 2). *In vivo*, blockade of T-cell co-stimulation around the time of engraftment was shown to allow for long-term allograft survival in several transplantation models (157). It has been demonstrated both *in vitro* and *in vivo* that blockade of the CD28/CTLA4:B7-1/B7-2 pathway in combination with the CD40L:CD40 pathway is more effective in the induction of antigen-specific tolerance than blockade of either pathway alone (158-162). Short-lived treatment of asthma patients that results in tolerance to the inhalant allergen and hence long-lasting improved clinical status would be a very attractive therapeutic approach. Therefore, in the study described in **chapter 5**, it was investigated if blockade of T-cell co-stimulation during the aerosol challenge period of OVA-sensitized mice results in long-term amelioration of asthma-like symptoms that is, not only immediately after the aerosol challenge period but also upon OVA re-challenge after serum clearance of experimental treatment (anti-CD40L mAb (MR1), murine CTLA4-IgG or the combination of these agents).

In the last chapter, the main results of the studies described in chapters 2-5 are summarized and discussed (**chapter 6**).

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Chapter 2

CD28/CTLA4 double deficient mice demonstrate crucial role for B7-1/B7-2 co-stimulation in allergic asthma

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ABSTRACT

The existence of a third B7-1/B7-2 receptor was postulated in a recent study using a novel mouse strain lacking both CD28 and CTLA4 (CD28/CTLA4$^{-/-}$) that demonstrated a substantial B7-1/B7-2-dependent Th1-mediated cardiac allograft rejection response, in contrast with long-term allograft survival in B7-1/B7-2$^{-/-}$ mice (1). In the present study, we investigated if T-cell co-stimulation *via* the putative B7-1/B7-2 receptor plays a role in the induction of Th2-mediated asthma-like symptoms in mice. BALB/c wild-type, CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice were sensitized and aerosol challenged with ovalbumin (OVA). At 24 h after the last aerosol, wild-type mice showed airway hyperresponsiveness *in vivo* to methacholine and up-regulated levels of serum OVA-specific IgE compared with the situation shortly before OVA challenge. In addition, eosinophils and IL-5 in the broncho-alveolar lavage fluid and Th2 cytokine production by lung cells upon OVA re-stimulation *in vitro* were observed. In agreement with an earlier study, we failed to induce any of the asthma-like symptoms in B7-1/B7-2$^{-/-}$ mice (2). Importantly, also CD28/CTLA4$^{-/-}$ mice showed no asthma-like symptoms upon OVA sensitization or challenge. These data clearly demonstrate that T-cell co-stimulation *via* the putative B7-1/B7-2 receptor appears to have no role in the induction of Th2-mediated asthma-like symptoms in this murine model and, conversely, that CD28 signaling is critical (3-5).

INTRODUCTION

Allergic asthma is a complex inflammatory disease of the airways characterized by reversible airflow obstruction, persistent airway hyperresponsiveness to bronchospasmogenic stimuli such as methacholine, chronic eosinophilic airway inflammation, and airway remodeling (6). Allergen-specific CD4+ type 2 helper T-(Th2) cells play a pivotal role in the pathogenesis and progression of allergic asthma by orchestrating the inflammatory response (7).

Over the past decade, it is well established that for optimal activation, T-lymphocytes require nonspecific co-stimulatory signals in addition to the antigen-specific signal conferred by the T-cell receptor (TCR) (8-10). Although many receptor-ligand pairs for T-cell co-stimulation have been identified since, CD28 is the primary co-stimulatory molecule, constitutively present on the surface of T-cells. Engagement of CD28 on naïve T-cells by its ligands B7-1 (CD80) or B7-2 (CD86) on antigen-presenting cells provides a potent co-stimulatory signal to T-cells activated through their TCR that contributes to elements of T-cell activation such as proliferation and IL-2 secretion. The second receptor for the B7-1/B7-2 ligands, CTLA4, is expressed on activated T-cells and delivers an inhibitory signal to terminate the T-cell response (11). In experimental murine models of allergic asthma, blockade of the CD28/CTLA4:B7-1/B7-2 pathway of T-cell co-stimulation during the antigen sensitization and/or challenge period ameliorates the asthma-like symptoms (12-17).

Interestingly, a recent study using a novel mouse strain lacking both CD28 and CTLA4 (CD28/CTLA4$^{-/-}$) provided evidence for the existence of an additional receptor for the B7-1 and B7-2 molecules (1). In this study, CD28/CTLA4$^{-/-}$ CD4+ T-cells demonstrated considerable residual B7-1/B7-2-dependent proliferation upon polyclonal stimulation *in vitro*. In addition, T-cell co-stimulation *via* this putative B7-1/B7-2 receptor was shown to contribute substantially to Th1-mediated cardiac allograft rejection *in vivo*, in contrast with long-term allograft survival in B7-1/B7-2$^{-/-}$ mice (18, 19).

The role of this putative B7-1/B7-2 receptor in other T-cell-mediated disease models is unknown at the moment. With regard to Th2-mediated allergic asthma, no asthma-like symptoms can be induced in B7-1/B7-2$^{-/-}$ mice (2) nor in wild-type mice treated with monoclonal antibodies (mAbs) against both B7-1 and B7-2 throughout the antigen sensitization and challenge period (4). In CD28$^{-/-}$ mice, however, the failure to induce manifestations of allergic asthma is accompanied by the presence of lymphocytes and IFN-γ in the broncho-alveolar lavage fluid (BALF) (4, 20) and the priming of lymphocytes in the lungs, spleen and lymph nodes (4, 5, 20). These data on residual T-cell activation in CD28$^{-/-}$ mice but not in B7-1/B7-2$^{-/-}$ mice (2), suggest a potential role for the putative B7-1/B7-2 receptor. Co-stimulation

by the supposed B7-1/B7-2 receptor would then result in allergen-specific T-cell activation that is not strong enough to induce the asthma-like symptoms. However, the failure to induce these symptoms in CD28$^{-/-}$ mice might also be due to negative signaling by CTLA4 (21-23) that opposes potential T-cell co-stimulation *via* the putative B7-1/B7-2 receptor. So, unmasking the possible role of co-stimulation by the supposed B7-1/B7-2 receptor in T-cell activation requires the absence of both CD28 and CTLA4.

Therefore, in the present study, we investigated if T-cell co-stimulation *via* the putative B7-1/B7-2 receptor contributes to the induction of Th2-dominated model of allergic asthma using the novel CD28/CTLA4$^{-/-}$ mouse strain. To this end, BALB/c wild-type mice, CD28/CTLA4$^{-/-}$ mice and B7-1/B7-2$^{-/-}$ mice were sensitized and aerosol challenged with the experimental allergen ovalbumin (OVA). At 24 h after the last OVA aerosol, wild-type mice show airway hyperresponsiveness to the non-specific stimulus methacholine, allergen-specific IgE in serum and an infiltration of eosinophils in the BALF. In addition, Th2-type cytokines were observed in the BALF and supernatant of OVA re-stimulated lung cell cultures. In agreement with an earlier study by Mark *et al*. (2), we failed to induce any of these asthma-like symptoms in the B7-1/B7-2$^{-/-}$ mice. Interestingly, also CD28/CTLA4$^{-/-}$ mice showed no manifestations of allergic asthma upon OVA sensitization and challenge. In addition, we were unable to demonstrate signs of residual OVA-specific CD4+ T-cell activation in CD28/CTLA4$^{-/-}$ mice. So, T-cell co-stimulation *via* the putative B7-1/B7-2 receptor appears to have no role in the induction of Th2-mediated asthma-like symptoms in this murine model. Conversely, further evidence is provided for the critical role of CD28 signaling in the development of Th2-dominated allergic inflammatory responses (3-5).

MATERIALS AND METHODS

Animals

Specific pathogen-free wild-type BALB/c mice were obtained from Charles River (Someren, The Netherlands). BALB/c CD28/CTLA4$^{-/-}$ mice (1) and BALB/c B7-1/B7-2$^{-/-}$ mice (24) were generated in the laboratory of Dr. A.H. Sharpe (Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA) which is an institution accredited by the American Association for the Accreditation of Laboratory Animal Care (AALAC). In the Netherlands, these mice were bred and maintained in a pathogen-free animal facility of the Central Laboratory Animal Institute (Utrecht).

Female and male (4:2) 12-13 wk-old mice were used for our studies. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments.

Antigen sensitization and challenge

All mice were sensitized and challenged with OVA (chicken egg albumin, crude grade V, Sigma, St. Louis, MO, USA). Systemic sensitization was performed by two intraperitoneal (i.p.) injections of 10 μg OVA adsorbed onto 2.25 mg aluminum hydroxide (alum; ImjectAlum, Pierce, Rockford, IL, USA) in 0.1 ml pyrogen-free saline on day 0 and 7. On day 35, 38 and 41, mice were challenged by inhalation of an OVA aerosol in a plexiglass exposure chamber (5 liter) for 20 min. The aerosols were generated by nebulizing an OVA solution (10 mg/ml in pyrogen-free saline) using a Pari LC Star nebulizer (PARI Respiratory Equipment, Richmond, VA, USA; particle size 2.5–3.1 μm) driven by compressed air at a flow rate of 6 liters/min.

Measurement of airway responsiveness in vivo

Airway responsiveness was measured in conscious, unrestrained mice on day 30 (5 d before the OVA aerosol challenge period) and at 24 h after the last OVA aerosol challenge on day 42, by recording respiratory pressure curves in response to inhaled nebulized methacholine (acetyl-β-methylcholine chloride; Sigma) using barometric whole-body plethysmography (Buxco; EMKA Technologies, Paris, France). Airway responses were expressed in enhanced pause (Penh), an index of airway obstruction as described in detail previously (25). Briefly, mice were placed in a whole-body chamber and pressure differences between this chamber and a reference chamber were recorded. After baseline Penh values were obtained for 3 min and averaged, animals were exposed to a saline aerosol and a series of methacholine aerosols (solutions doubling in concentration, ranging from 1.56–50 mg/ml in saline). Aerosols were generated by a Pari LC Star nebulizer for 3 min and after each nebulization readings were taken for 3 min and averaged.

Determination of OVA-specific IgE levels in serum

At day –3 and day 32 (3 d before challenge), approximately 0.25 ml blood was recovered from mice by an incision the tail vein. After measurement of airway responsiveness *in vivo* at 24 h after the last OVA aerosol challenge on day 42, mice were sacrificed by i.p. injection of 1 ml 10% urethane (Sigma) in sterile saline and bled by cardiac puncture. Serum was prepared from blood samples and stored at -20°C until determination of OVA-specific IgE levels by ELISA. Briefly, maxisorp 96-wells flat-bottomed microtiter plates (Nunc A/S, Roskilde, Denmark) were coated overnight at 4°C with 1 μg/ml rat anti-mouse IgE mAb (clone R35-72, PharMingen, San Diego, CA, USA) diluted in phosphate-buffered saline (PBS). The next day, the ELISA was performed at room temperature using ELISA buffer (PBS containing 0.5% bovine serum albumin [BSA, Sigma], 2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.05% Tween-20 [Merck, Whitehouse Station, NJ, USA], pH 7.2) for blocking and dilution of samples, standard and reagents and PBS containing 0.05% Tween-20

for washing between incubations. After blocking of wells for 1 h, serum samples and a duplicate dilution series of an OVA-specific reference serum (starting 1:40) were added to the wells and incubated for 2 h. An OVA-specific IgE reference serum was obtained by sensitization and challenge of mice with OVA as above-decribed and arbitrarily assigned a value of 1,000 experimental units/ml (EU/ml). Hereafter, 1 μg/ml of OVA labeled to digoxigenin (DIG) by a DIG protein labeling kit (Roche Diagnostics, Basel, Switzerland) was added for 1.5 h, followed by incubation with 1:500 diluted anti-DIG-Fab fragments coupled to horseradish peroxidase (Roche Diagnostics) for 1 h. Color development was performed with *o*-phenylenediamine-dichloride substrate (0.4 mg/ml, Sigma) and 4 mM H_2O_2 in PBS and the reaction was stopped by adding 4 M H_2SO_4. The optical density was read at 490 nm, using a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Results were analyzed with Microplate Manager PC software (Bio-Rad Laboratories). The lower detection limit of the ELISA was 4 EU/ml IgE.

Analysis of the BALF

BAL was performed immediately after bleeding of the mice as described in detail previously (26). Briefly, the airways were lavaged through a tracheal cannula with 1 ml saline at 37°C containing 5% BSA and 2 μg/ml aprotinine (Roche Diagnostics). The recovered lavage fluid of this first ml was kept apart for determination of the amount of IL-5 in the supernatant by ELISA. Subsequently, mice were lavaged four times with 1 ml aliquots of saline only at 37°C. Recovered lavage fluid of the second through fifth ml was pooled and cells (including those from the first ml) were pelleted (387 × *g*, 4°C, 10 min), and resuspended in 0.15 ml cold PBS. The total number of cells in the BALF was determined using a Bürker-Türk counting-chamber (Karl Hecht Assistent KG, Sondheim/Röhm, Germany). For differential BALF cell counts, cytospin preparations were made (15 × *g*, 4°C, 5 min) using a cytocentrifuge (Shandon Life Science, Cheshire, UK), and cells were fixed and stained with Diff-Quick (Dade A.G., Düdingen, Switzerland). Per cytospin, at least 200 cells were counted and differentiated into mononuclear cells (monocytes, macrophages and lymphocytes), eosinophils and neutrophils by standard morphology and staining characteristics. The investigator counting the cells was blinded to the treatment groups.

Culture of lung cells

Lungs were lavaged as above-described and perfused *via* the right heart ventricle with 4 ml saline at 37°C containing 100 U/ml heparin (Leo Pharmaceuticals, Weesp, The Netherlands) to remove any blood and intravascular leukocytes. Complete lung tissue was removed and transferred to cold sterile PBS. Lungs were then minced and digested in 3 ml RPMI 1640 containing 2.4 mg/ml collagenase A and 1.0 mg/ml DNase I (grade II) (both from Roche Diagnostics) for 30 min at 37°C. Enzyme

activity was stopped by adding 1 ml fetal calf serum (FCS). The lung tissue digest was filtered through a 70-µm nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA) with 10 ml RPMI 1640 to obtain a single-cell suspension. The lung-cell suspension was washed, resuspended in culture medium (RPMI 1640 supplemented with 10% FCS, 1% glutamax I and gentamicin [both from Life Technologies, Gaithersburg, MD, USA] and 50 µM β-mercaptoethanol [Sigma]), and the total number of lung cells was determined using a Bürker-Türk counting-chamber. Lung cells (8×10^5 lung cells/well) were cultured in 96-well round-bottomed plates (Greiner Bio-One, Kremsmuenster, Austria) in the presence or absence of OVA (10 µg/ml). In addition, lung cells were polyclonally stimulated with plate-bound rat anti-mouse CD3 mAb (clone 17A2 rIgG2b, 50 µg/ml, coated overnight at 4°C). The hybridoma for the 17A2 mAb to CD3 was obtained from the American Type Tissue Collection, Manassas, VA, USA) and purified Ab was used. After 5 d of culture at 37°C in 5% CO_2, the supernatant was harvested, and stored at -20°C until determination of cytokine levels by ELISA.

Measurement of cytokines

Supernatants of the first ml BALF and lung cell-cultures were analyzed for IFN-γ, IL-5 and IL-10 content by sandwich ELISA using Ab pairs and standards purchased from PharMingen, according to the manufacturer's instructions. The lower detection limits of the ELISAs were 16 pg/ml for IFN-γ, 32 pg/ml for IL-5, and 63 pg/ml for IL-10.

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). The airway dose-response curves to methacholine were statistically analyzed by a general linear model of repeated measurements followed by *post-hoc* comparison between groups. Data were log10 transformed before analysis to equalize variances in all groups. Statistical analysis on BALF cell counts was performed using the non-parametric Mann Whitney *U* test. For all other parameters, results were statistically analyzed using a Student's *t* test (two-tailed, homosedastic). Differences between groups were considered statistically significant at the $p < 0.05$ level. Statistical analyses were performed using SPSS for Windows version 10.0.5 (SPSS, Chicago, IL, USA).

RESULTS

BALB/c wild-type, CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice were i.p. sensitized to 10 µg OVA adsorbed to alum on day 0 and 7 and aerosol challenged with a 1.0% OVA solution for 20 min on day 35, 38 and 41.

Absence of OVA-specific IgE in the sera from CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice
To determine allergen-specific IgE levels in serum, blood was withdrawn from each mouse before sensitization to OVA (pre-serum), after sensitization but before the OVA aerosol challenge period (post-sensitization serum) and at 24 h after the last OVA aerosol (final serum). In the pre-sera from the three BALB/c strains, no OVA-specific IgE could be detected. After sensitization to OVA, antigen-specific IgE was found in the sera from wild-type mice (11,664 ± 2,034 EU/ml) and these levels were greatly up-regulated upon OVA aerosol challenge (103,487 ± 15,811 EU/ml, $p < 0.0005$). In contrast, in both the post-sensitization- and final sera from CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice the amount of OVA-specific IgE was below the detection level of the ELISA (4 EU/ml).

These data suggest that no sensitization to OVA had taken place in the animals deficient in CD28 and CTLA4 or the B7 molecules.

Absence of airway hyperresponsiveness in CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice
Airway responsiveness *in vivo* to methacholine was determined in conscious, unrestrained mice by barometric whole-body plethysmography and was first measured after sensitization to OVA but before the aerosol challenge period. As shown in Figure 1, all three OVA-sensitized BALB/c strains, demonstrated a dose-dependent increase in Penh, an index of airway obstruction, in response to aerosolized methacholine and the complete methacholine dose-response curves (DRCs) did not differ between the strains (p =1 for each combination of two strains). Airway responsiveness *in vivo* was measured again at 24 h after the last OVA aerosol.

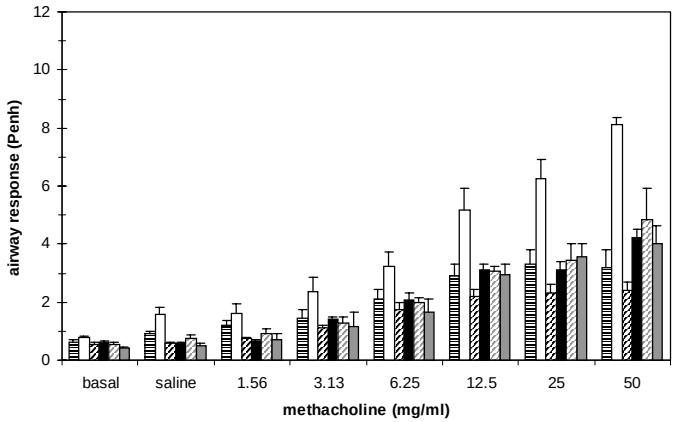

Figure 1: Absence of airway hyperresponsiveness in CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice.
BALB/c wild-type (*first and second bar*), CD28/CTLA4$^{-/-}$- (*third and fourth bar*) and B7-1/B7-2$^{-/-}$ mice (*fifth and sixth bar*) were sensitized to OVA adsorbed to alum and challenged with three OVA aerosols. Airway responsiveness *in vivo* to aerosolized methacholine was measured in conscious, unrestrained mice by whole-body plethysmography before (*striped bars*) and at 24 h after (*plain bars*) the OVA aerosol challenge period. Values are expressed as mean ± SEM (n = 5-6 per group).

Upon OVA aerosol challenge, wild-type mice developed airway hyperresponsiveness because the second methacholine DRC is significantly different from the DRC obtained before challenge in this group of mice ($p < 0.05$). In contrast, airway responsiveness to methacholine before- and at 24 h after the OVA aerosol challenge period was not different in CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice, when comparing the complete DRCs ($p = 1$ and $p = 1$, respectively). Comparing the airway responses to the separate concentrations of the methacholine DRC before- and after OVA challenge, CD28/CTLA4$^{-/-}$ mice showed airway hyperresponsiveness to 50 mg/ml methacholine ($p < 0.05$) (Figure 1). However, in a duplicate experiment, CD28/CTLA4$^{-/-}$ mice demonstrated no induction of hyperresponsiveness upon OVA challenge to each separate concentration of the methacholine DRC (data not shown).

So, no residual airway hyperresponsiveness was induced in mice lacking both CD28 and CTLA4 upon OVA-sensitization and challenge.

Absence of eosinophils and IL-5 in the BALF from CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice

The number of various leukocytes in the BALF was used as a measure for the infiltration of these cells in the airways and was determined at 24 h after the last OVA aerosol challenge. OVA sensitization and challenge from wild-type mice resulted in an influx of inflammatory cells in the BALF, predominantly eosinophils besides mononuclear cells (monocytes, macrophages and lymphocytes) and a few neutrophils (Figure 2). In contrast, as in naïve wild-type mice (data not shown), only mononuclear cells were present in the BALF from CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice upon OVA-sensitization and challenge and the number of mononuclear cells was significantly less compared with OVA-sensitized and challenged wild-type mice ($p < 0.005$ and $p < 0.05$, respectively).

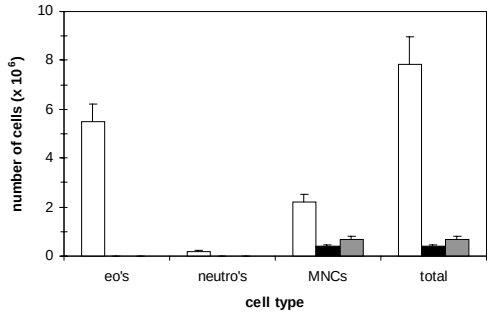

Figure 2: Absence of eosinophils in the BALF from CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice. BALB/c wild-type (*open bars*), CD28/CTLA4$^{-/-}$ (*closed bars*) and B7-1/B7-2$^{-/-}$ mice (*shaded bars*) were sensitized to OVA adsorbed to alum and challenged with three OVA aerosols. At 24 h after the last OVA aerosol challenge, BALF was recovered and the cellular composition was determined. Values are expressed as mean ± SEM (n = 5-6 per group).

The influx of inflammatory cells in the BALF of OVA-sensitized and challenged wild-type mice was accompanied by the presence of IL-5 (0.67 ± 0.08 ng/ml), but no detectable levels of IFN-γ, pointing to a Th2-dominated response. Because IL-5 is involved in the migration of eosinophils, this is in accordance with the BALF eosinophilia observed in this group of mice. In contrast, no IL-5 (and no IFN-γ) could be detected in the BALF from CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice.

So, no residual Th2-dominated inflammatory response could be observed in the BALF from CD28/CTLA4$^{-/-}$ mice upon OVA-sensitization and challenge.

Lung T-cells from CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice are not primed

A single-cell suspension of lung tissue was prepared at 24 h after the last OVA aerosol. To determine antigen-specific T-cell cytokine responses, lung cells were re-stimulated with 10 μg/ml OVA for 5 d. In addition, lung T-cells were polyclonally stimulated with 50 μg/ml anti-CD3 mAb to determine their intrinsic capacity to produce cytokines or cultured with medium only.

Lung cell cultures derived from OVA-sensitized and challenged wild-type mice only produced a small amount of IFN-γ upon stimulation with anti-CD3 mAb (0.042 ± 0.014 ng/ml) (Figure 3) and this amount was reduced compared with cultures from naïve wild-type mice (0.128 ± 0.021 ng/ml; data not shown in Figure 3). However, these cultures produced fair amounts of the Th2 cytokines IL-5 and IL-10 upon re-stimulation with OVA (8.20 ± 1.35 ng/ml and 4.73 ± 1.48 ng/ml, respectively) and polyclonal stimulation (7.97 ± 1.45 ng/ml and 3.31 ± 0.71 ng/ml, respectively). So, OVA-specific T-cells were present in lung tissue derived from OVA-sensitized and challenged wild-type mice that showed a clear Th2-response upon antigenic re-stimulation *in vitro.*

Polyclonal stimulation of lung cell cultures derived from CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice sensitized and challenged with OVA, resulted in significantly more IFN-γ production (0.52 ± 0.09 ng/ml, p <0.005 and 0.39 ± 0.13 pg/ml, p <0.05, respectively) compared with cultures from OVA-sensitized and challenged wild-type mice (0.042 ± 0.014 ng/ml). In contrast, significantly less IL-10 could be observed in the supernatant of these cultures after 5 d of stimulation with anti-CD3 mAb (1.09 ± 0.12 ng/ml, p < 0.01 and 0.69 ± 0.17 pg/ml, p < 0.01, respectively), compared with those from OVA-sensitized and challenged wild-type mice (3.31 ± 0.71 ng/ml). IL-5 could only be detected in the supernatant of cultures derived from CD28/CTLA4$^{-/-}$ mice (0.94 ± 0.25 ng/ml) and this amount was significantly less compared with cultures from OVA-sensitized and challenged wild-type mice stimulated with anti-CD3 mAb (7.97 ± 1.45 ng/ml, p < 0.001). Antigenic re-stimulation of lung cell cultures from OVA-sensitized and challenged CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice did not result in cytokine production above medium control values.

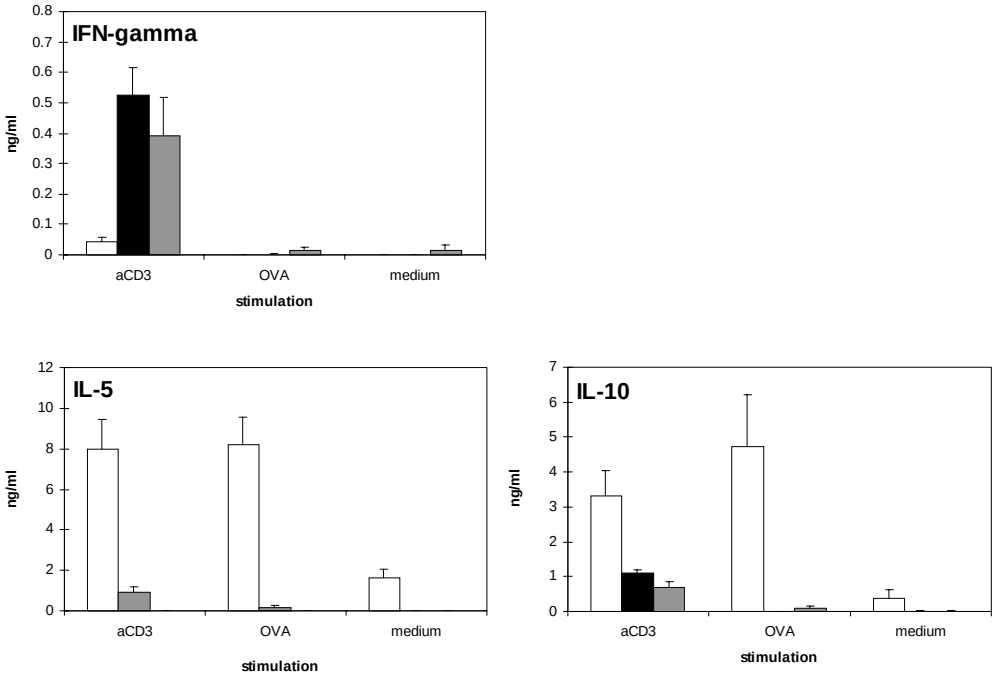

Figure 3: Lung T-cells from CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice are not primed.
BALB/c wild-type (*open bars*), CD28/CTLA4$^{-/-}$ (*closed bars*) and B7-1/B7-2$^{-/-}$ mice (*shaded bars*) were sensitized to OVA adsorbed to alum and challenged with three OVA aerosols. At 24 h after the last OVA aerosol challenge, a single-cell suspension of lung tissue was prepared. Lung cells were stimulated for 5 d with 50 μg/ml anti-CD3 mAb or 10 μg/ml OVA or cultured with medium only and IFN-γ, IL-5 and IL-10 in the supernatant of the cultures was determined. Values are expressed as mean ± SEM (n = 5-6 per group).

So, these data suggest that T-cells present in lung tissue derived from CD28/ CTLA4-/- and B7-1/B7-2-/- mice were not primed after sensitization and challenge with OVA *in vivo*, as determined by undetectable cytokine production upon OVA re-stimulation *ex vivo*. Interestingly, these unprimed T-cells seemed to be characterized by an enhanced IFN-γ reactivity because these cultures produced significantly more IFN-γ upon polyclonal stimulation not only compared with wild-type mice sensitized and challenged with OVA but also in comparison with naïve wild-type mice (data not shown in Figure 3).

DISCUSSION

A recent study using a novel mouse strain lacking both CD28 and CTLA4 demonstrated considerable residual CD4+ T-cell proliferation *in vitro* that was

dependent on B7-1 or B7-2, suggesting the existence of an additional third B7-1/B7-2 receptor (1). In addition, CD28/CTLA4$^{-/-}$ mice mounted a substantial Th1-mediated cardiac allograft rejection response *in vivo*, in contrast with long-term allograft survival in B7-1/B7-2$^{-/-}$ mice (18, 19). The role of this putative B7-1/B7-2 receptor in other T-cell-mediated disease models is unknown at the moment. Therefore, in the present study, we investigated if T-cell co-stimulation *via* the supposed B7-1/B7-2 receptor has a role in the induction of Th2-dominated murine model of allergic asthma. Sensitization and aerosol challenge of wild-type mice with OVA resulted in airway hyperresponsiveness to methacholine and the influx of eosinophils in the BALF, cardinal airway features of patients suffering from allergic asthma. In addition, OVA-specific IgE in serum, IL-5 in the BALF and Th2 cytokine production by lung cells upon OVA re-stimulation *in vitro* were observed. In agreement with an earlier study (2), we failed to induce any of the asthma-like symptoms in the B7-1/B7-2$^{-/-}$ mice. Interestingly, also CD28/CTLA4$^{-/-}$ mice showed no manifestations of allergic asthma upon OVA sensitization and challenge. So, these data clearly demonstrate that T-cell co-stimulation *via* the putative B7-1/B7-2 receptor appears to have no role in the induction of Th2-mediated asthma-like symptoms in this murine model. Conversely, these data provide further evidence for the critical role of CD28 signaling in the development of Th2-dominated allergic inflammatory responses (3-5).

Previously, it has been demonstrated that no asthma-like symptoms can be induced in CD28$^{-/-}$ mice (4, 5, 20). In these mice, however, the contribution of other molecules including the putative B7-1/B7-2 receptor to T-cell co-stimulation might be masked by negative signaling *via* CTLA4 (21-23). Following the observation that no manifestations of allergic asthma could be induced in CD28$^{-/-}$ mice, two research groups administered CTLA4-Ig to these mice (4, 5) to determine whether the absence of asthma-like symptoms was due exclusively to the lack of a positive signal by CD28 or whether an unopposed negative signal *via* CTLA4 was inhibiting the T-cell response. Incongruent results were obtained because in one study CTLA4-Ig administration had no effect (4), whereas treatment of CD28$^{-/-}$ mice with CTLA4-Ig restored lymphocyte but not eosinophil recruitment to the airways in the second study (5).

Importantly, CTLA4-Ig prevents the interaction of the B7-1 and B7-2 molecules with both CTLA4 and the putative B7-1/B7-2 receptor, thus not allowing for discrimination between these two pathways regulating T-cell activation. In addition, treatment of mice with CTLA4-Ig might leave some B7-1 and B7-2 molecules uncovered (27) that may bind CTLA4 (and the supposed B7-1/B7-2 receptor), whereas deficiency of a gene encoding a particular molecule guarantees the complete absence of this molecule. Therefore, unraveling the potential role of co-stimulation by the putative B7-1/B7-2 receptor required the absence of both CD28 and CTLA4. This study demonstrates that the additional deficiency of the CTLA4 molecule in mice

did not result in considerable residual manifestations of allergic asthma by unmasking co-stimulation *via* the supposed B7-1/B7-2 receptor.

In CD28$^{-/-}$ mice, the failure to induce manifestations of allergic asthma is accompanied by signs of residual T-cell activation; lymphocytes and IFN-γ are present in the BALF (4, 20) and lymphocytes obtained from the lungs, spleen and lymph nodes respond to antigenic re-stimulation *in vitro* (4, 5, 20). Likewise, the absence of the key airway features in CD28/CTLA4$^{-/-}$ mice might leave open the possibility that OVA-specific CD4+ T-cells in these mice are in fact co-stimulated *via* the putative B7-1/B7-2 receptor, resulting in T-cell activation that is not strong enough to induce airway pathology. However, the data presented in this report provide no evidence for residual T-cell activation in CD28/CTLA4$^{-/-}$ mice. First, the amount of antigen-specific IgE in serum after OVA immunization was below the detection level, suggesting that no or insufficient systemic Th2 cells have developed because B-cells require help by systemic Th2 cells for isotype switching to IgE (28). It should be noted, that airway hyperresponsiveness *in vivo* and airway eosinophilia can be induced in mice in the absence of systemic IgE (29) but has been shown to be critically dependent on CD4+ cells (30-33). Second, T-cells present in lung tissue derived from CD28/CTLA4$^{-/-}$ mice produced no cytokines upon antigenic re-stimulation *in vitro*, indicating that they were not primed upon OVA sensitization and challenge *in vivo*.

Yet it remained possible, that OVA-specific CD28/CTLA4$^{-/-}$ CD4+ cells in the lung draining lymph nodes were co-stimulated *via* the putative B7-1/B7-2 receptor but had failed to migrate to the airways. To test this hypothesis, also the thoracic lymph nodes (TLNs) were isolated at 24 h after the last OVA aerosol challenge in the presented experiment. Whereas wild-type, CD4+ lymphocytes produced a small amount of IFN-γ and fair amounts of IL-4, IL-5 and IL-10 upon polyclonal stimulation *in vitro*, no cytokines were detectable in the supernatant of CD4+ TLN cell cultures derived from both CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice (data not shown). In addition, the percentage of CD4+ cells in the TLN was very similar in OVA-sensitized and challenged CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice, indicating that no residual CD4+ T-cell expansion had occurred in CD28/CTLA4$^{-/-}$ mice. Finally, FACS analysis demonstrated no enhanced expression of activation markers such as CD25, CD62L and CD69 on CD4+ TLN cells obtained from CD28/CTLA4$^{-/-}$ mice in comparison with B7-1/B7-2$^{-/-}$ mice (data not shown). In conclusion, we were unable to demonstrate any sign of residual T-cell activation in both the TLNs and lungs from CD28/CTLA4$^{-/-}$ mice upon OVA-sensitization and challenge *in vivo*.

The discrepancy of residual T-cell activation in CD28$^{-/-}$ mice (4, 5, 20) versus no signs of T-cell activation in CD28/CTLA4$^{-/-}$ mice in this experiment might be explained by the different antigens and protocols used for the induction of manifestations of allergic asthma. For example, Mathur *et al*. used eggs and antigen

of the *Schistosoma mansoni* parasite (4) and Lambrecht *et al*. passively sensitized naïve CD28$^{-/-}$ mice with OVA-pulsed myeloid dendritic cells (20).

Antigenic re-stimulation of lung cell cultures from OVA-sensitized and challenged CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice did not result in Th1 or Th2 cytokine production above medium control values. Interestingly, the seemingly unprimed T-cells from both OVA-sensitized and challenged CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice were characterized by an enhanced IFN-γ reactivity. Lung cell cultures derived from these mice produced more IFN-γ upon polyclonal stimulation, not only compared with wild-type mice displaying Th2-mediated asthma-like symptoms upon OVA sensitization and challenge but also compared with naïve wild-type mice. In agreement herewith, BALF CD4+ T-cells obtained after challenge from passively sensitized CD28$^{-/-}$ mice were shown to have an increased tendency to produce IFN-γ upon polyclonal stimulation compared with wild-type mice (20). Moreover, CD4+ CD28/CTLA4$^{-/-}$ cells produced more IFN-γ and less IL-4 and IL-10 upon secondary polyclonal stimulation *in vitro* compared with wild-type CD4+ cells (1). These findings are consistent with the importance of CD28 for Th2 differentiation (3). In addition, this observation might explain why it is seems impossible to prime antigen-specific T-cells towards the Th2 phenotype -as opposed to a Th1-mediated cardiac allograft rejection (1, 19, 23, 34)- even though a protocol was used that normally results in very strong Th2-mediated inflammatory responses in BALB/c mice. However, the enhanced IFN-γ reactivity did not prime OVA-specific T-cells towards the Th1 phenotype either as observed locally in lung T-cell re-stimulation and systemically by the absence of the Th1 cell-dependent IgG2a isotype (35). In wild-type mice, besides a high level of IgE also OVA-specific IgG2a can be detected upon sensitization and challenge with OVA (data not shown).

Apparently, the putative B7-1/B7-2 receptor has a differential role in Th1-mediated cardiac allograft rejection and Th2-mediated allergic asthma. Perhaps, the expression of the supposed B7-1/B7-2 receptor is differentially regulated on Th1 versus Th2 cells. Indeed, T helper cells can be divided not only on the basis of their cytokine profile but also on their differential expression (kinetics) of cell surface molecules such as chemokine receptors and the CD28-related T-cell co-stimulator (ICOS) (36-38). Future studies regarding the induction of other T-cell-mediated diseases in CD28/CTLA4$^{-/-}$ mice might help to clarify the precise role of the supposed B7-1/B7-2 receptor in T-cell responses. Ultimately, cloning of the gene encoding the putative B7-1/B7-2 receptor will greatly facilitate research on this receptor.

Whereas CD28$^{-/-}$ mice are still capable of raising a number of *in vivo* immune responses, including cardiac allograft rejection (1, 19, 23, 34), induction of allogeneic graft-versus-host disease (39), generation of cytotoxic T-cells (40) and certain delayed-type hypersensitivity responses (41), the present study provides further evidence for the critical role of CD28 signaling in the development of Th2-dominated

allergic inflammatory responses (3-5, 20). Moreover, as anticipated by the data on the failure to induce asthma-like symptoms in B7-1/B7-2$^{-/-}$ mice (Ref. 2 and this study), T-cell co-stimulatory receptors that interact with other ligands than B7-1 and B7-2, *e.g.* ICOS, appear to play no major role in the induction of asthma manifestations in this model. So, blockade of CD28 in allergic asthma appears to be a very promising therapeutic option.

ACKNOWLEDGEMENTS

D.T.D and E.C.A.M.v.E are supported by Research Grant 3.2.97.09 from The Netherlands Asthma Foundation.

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Chapter 3

CTLA4-IgG reverses asthma manifestations in a mild- but not in a more “severe” ongoing murine model

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Adapted from: *Am J Respir Cell Mol Biol* 2001;25(12):751-60

ABSTRACT

We investigated whether CTLA4-Ig can reverse established asthma manifestations in a novel murine model of ongoing disease. In BALB/c mice, sensitized to ovalbumin (OVA) without adjuvant, airway inflammation was induced by a first series of OVA aerosol challenges. Murine CTLA4-IgG was then administered, followed by a second series of OVA inhalations. In control-treated mice, two series of OVA challenges induced up-regulation of OVA-specific IgE in serum, eosinophils in the broncho-alveolar lavage fluid (BALF) and IL-5 production by lung lymphocytes upon OVA re-stimulation *in vitro*, compared with saline-challenged mice. CTLA4-IgG significantly inhibited all of these parameters in OVA-challenged mice. Importantly, mCTLA4-IgG performed better than the gold-standard dexamethasone because this corticosteroid did not inhibit the up-regulation of OVA-specific IgE in serum. In a more "severe" ongoing model, induced by sensitization to OVA emulsified in aluminum hydroxide, resulting in airway hyperresponsiveness to methacholine and stronger inflammatory responses, mCTLA4-IgG was less effective in that only the number of eosinophils in the BALF was reduced ($p = 0.053$), whereas dexamethasone inhibited both BALF eosinophilia and cytokine production by lung lymphocytes. So, CTLA4-Ig might be an effective alternative therapy in established allergic asthma, especially in situations of mild disease.

INTRODUCTION

Allergic asthma is prevalent worldwide, but especially in developed countries its prevalence is increasing to epidemic proportions (1). Patients with asthma suffer from acute broncho-constriction and mucus formation directly after inhalation of the allergen. Chronic symptoms include
airway hyperresponsiveness to broncho-spasmogenic stimuli, inflammation and airway remodeling.
CD4+ T helper cells recognizing the allergenic peptides and differentiating into the type 2 subset of CD4+ T helper cells (Th2 cells), are crucial for the initiation and progression of allergic asthma (2). Th2 cells are characterized by the array of cytokines they secrete, which are optimized to combat parasite infections (3). However, Th2 cells are developed undesirably in allergic disorders such as asthma, their cytokines causing airway symptoms either directly or mediated by other immune cells and their mediators (4).

In view of the essential role of Th2 cells in allergic asthma, these cells are interesting target cells for therapy. One therapeutic strategy is the prevention of optimal activation of allergen-specific CD4+ T-cells by blocking their co-stimulatory requirements. For complete activation, CD4+ T-cells need nonspecific co-stimulatory signals in addition to the signal provided by the T-cell receptor (TCR) after interacting with the MHC class II/antigenic peptide complex on the same antigen-presenting cell. CD28 is the primary T-cell co-stimulatory molecule, constitutively present on the surface of T-cells. Upon interaction with its ligands B7-1 (CD80) and/or B7-2 (CD86), CD28 tranduces a signal that enhances T-cell proliferation and cytokine secretion and sustains the T-cell response. The second receptor for the B7 ligands, CTLA4 (CD152) is detectable on the cell surface 24 h after T-cell activation *in vitro* and *in vivo* and down-regulates T-cell responses. Because CTLA4 has a higher affinity for the B7 ligands compared with CD28, CTLA4-Ig has been widely used to block T-cell co-stimulation in an array of experimental animal models of T-cell-mediated diseases (5).

Previous studies have shown that blockade of T-cell co-stimulation by administration of CTLA4-Ig ameliorates the asthmatic manifestations in murine models (6-10). In all of these studies, CTLA4-Ig was administered before the onset of asthma manifestations. However, asthmatic patients start therapy after the onset of T-cell-mediated airway symptoms, while exposure to environmental allergen very often continues. In addition, it has been shown that memory- and recently activated effector T-cells are less dependent on CD28-mediated co-stimulation for their activation than naïve T-cells (11-14). This might limit the therapeutic potential of CTLA4-Ig when administered after the development of T-cell-mediated airway symptoms in an ongoing disease.

To more closely resemble our adjuvant-free, ovalbumin (OVA)-induced animal model of allergic asthma (15, 16) to the clinical situation, we designed a new protocol in which treatment starts after the onset of airway inflammation while OVA exposures continue. In the present study, we applied murine CTLA4-IgG in this model of ongoing disease to determine if co-stimulatory blockade is also effective after the onset of T-cell-mediated airway inflammation. We are the first to demonstrate that, in an ongoing murine model of allergic inflammation, mCTLA4-IgG significantly inhibited the up-regulation of OVA-specific IgE in serum, the number of eosinophils in broncho-alveolar lavage fluid (BALF) and IL-5 production by lung lymphocytes *in vitro*. Importantly, mCTLA4-IgG performed better than the gold-standard dexamethasone because this corticosteroid did not inhibit the up-regulation of OVA-specific IgE in serum. In a more "severe" ongoing model, induced by sensitization to OVA emulsified in aluminum hydroxide (alum) resulting in airway hyperresponsiveness and stronger inflammatory responses, mCTLA4-IgG was less effective in that only the number of eosinophils in the BALF was reduced ($p = 0.053$), whereas dexamethasone inhibited both BALF eosinophilia and cytokine production by lung lymphocytes.

MATERIALS AND METHODS

Animals

Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Specific pathogen-free male BALB/c mice (6 wk old) were obtained from the Central Laboratory Animal Institute (Utrecht, The Netherlands). The mice were housed in macrolon cages in a laminar flow cabinet and provided with food and water *ad libitum*.

Sensitization and challenge

All mice were sensitized to OVA (chicken egg albumin, crude grade V, Sigma, St. Louis, MO). Active sensitization was performed without an adjuvant by giving seven intraperitoneal (i.p.) injections of 10 μg OVA in 0.5 ml pyrogen-free saline on alternate days (one injection per day). Three weeks after the last sensitization, mice were exposed to either 16 OVA challenges (2 mg/ml in pyrogen-free saline) or 16 saline aerosol challenges for 5 min on consecutive days (one aerosol per day). An additional group of mice first received eight OVA aerosols, followed by eight saline aerosols (OVA/saline, "spontaneous resolution group") (*see* Table 1).

For the experiment in the more "severe" ongoing model, all mice were sensitized to OVA by active sensitization with two i.p. injections (7 d apart) of 0.1 ml alum-precipitated antigen, comprising 10 μg OVA adsorbed onto 2.25 mg alum (ImjectAlum; Pierce, Rockford, IL). Two weeks after the second sensitization, mice

were exposed to either six OVA challenges (10 mg/ml in pyrogen-free saline) or six saline aerosol challenges for 20 min every third day (one aerosol every third day). An additional group of mice first received three OVA aerosols, followed by three saline aerosols (OVA/saline, "spontaneous resolution group") (*see* Table 2).

The aerosol was performed in a plexiglas exposure chamber (5 liter) coupled to a Pari LC Star nebulizer (PARI Respiratory Equipment, Richmond, VA; particle size 2.5–3.1 μm) driven by compressed air at a flow rate of 6 liters/min. Aerosol was given in groups composed of no more than eight animals.

Experimental treatment protocols

Both saline and OVA-challenged mice were divided in two groups of eight mice. One group of mice received experimental treatment, and the other group received control treatment. The spontaneous resolution group received control treatment in both independent experiments.

In experiment A, murine CTLA4-IgG fusion protein was used as the experimental treatment, consisting of murine CTLA4 fused via an ECD-immunoglobulin junction to the hinge and Fc regions of human IgG1 (9). Human total IgG was used as control treatment in this experiment. Ten minutes before the ninth aerosol, mice were intravenously (i.v.) injected with 110 μl saline containing either 143 μg mCTLA4-IgG or human total IgG. Ten minutes before each of the seven following aerosols, mice were subcutaneously injected with 72 μg of the antibodies in a volume of 55 μl. In experiment B, dexamethasone (1:40 diluted in saline) was used as experimental treatment and saline as control treatment. Ten minutes before the ninth through the sixteenth aerosol, mice were injected i.p. with 0.0125 mg dexamethasone in 0.25 ml pyrogen-free saline or saline alone (*see* Table 1).

In the experiment using the more "severe" ongoing model, the group of saline-challenged mice ($n = 8$) and the spontaneous resolution group ($n = 8$) received control treatment. OVA-challenged animals were divided into three groups of eight animals each, receiving either control treatment, murine CTLA4-IgG fusion protein or dexamethasone. Ten minutes before the fourth aerosol, a single dose of control treatment (human total IgG, 280 μg in 0.2 ml saline) or murine CTLA4-IgG fusion protein (280 μg in 0.2 ml saline) was i.v. administered. This single dose resulted in very similar concentrations of the antibodies in the final sera as observed in those from the experiment in the milder ongoing model of airway inflammation. Dexamethasone treatment (0.0125 mg in 0.25 ml pyrogen-free saline) was started 10 min before the fourth aerosol and was followed by six daily i.p. injections (one injection per day) during the period of the second series of OVA aerosols (*see* Table 2).

Human total IgG was purchased from ICN Pharmaceuticals (Costa Mesa, CA) and dissolved in saline. This solution was treated with 10% (v/v) polymyxin B-

agarose (Sigma) for 1 h at 4°C to remove lipopolysaccharide. After incubation, the agarose-beads were removed by centrifugation and the supernatant was sterilized. Murine CTLA4-IgG fusion protein was kindly provided by Dr. P. Jardieu (Immunology, Genentech, South San Francisco, CA) and constructed as described previously (9). The endotoxin level was less than 1 endotoxin U/mg. Dexamethasone (Dexadreson) was purchased from Intervet Nederland BV (Boxmeer, The Netherlands).

In all experiments, airway responsiveness to methacholine, OVA-specific IgE levels in serum, cellular infiltration in the BALF and T-cell responses in lung tissue were measured 24 h after the last challenge in each mouse.

Measurement of airway responsiveness in vivo

Airway responsiveness was measured in conscious, unrestrained mice using barometric whole-body plethysmography by recording respiratory pressure curves (Buxco; EMKA Technologies, Paris, France) in response to inhaled methacholine (acetyl-β-methylcholine chloride; Sigma). Airway responsiveness was expressed in enhanced pause (Penh), as described in detail previously (17). Briefly, mice were placed in a whole-body chamber and basal readings were obtained and averaged for 3 min. Aerosolized saline, followed by doubling concentrations of methacholine (ranging from 1.6–50 mg/ml saline), were nebulized for 3 min and readings were taken and averaged for 3 min after each nebulization. Dose-response curves to methacholine were statistically analyzed by a general linear model of repeated measurements followed by *post-hoc* comparison between groups. Data were LOG transformed before analysis to equalize variances in all groups.

Determination of OVA-specific IgE levels in serum

After measurement of *in vivo* airway responsiveness, mice were sacrificed by i.p. injection of 1 ml 10% urethane in pyrogen-free saline (Sigma). Subsequently, mice were bled by cardiac puncture and OVA-specific IgE was measured by ELISA. Briefly, microtiter plates (Nunc A/S, Roskilde, Denmark) were coated overnight at 4°C with 2 μg/ml rat anti-mouse IgE monoclonal antibody (mAb) (clone EM95) diluted in phosphate-buffered saline (PBS). The next day, the ELISA was performed at room temperature. After blocking with ELISA buffer (PBS containing 0.5% bovine serum albumin [Sigma], 2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.05% Tween-20 [Merck, Whitehouse Station, NJ], pH 7.2) for 1 h, serum samples and a duplicate standard curve (starting 1:10), diluted in ELISA buffer, were added for 2 h. An OVA-specific IgE reference standard was obtained by i.p. immunization with OVA and arbitrarily assigned a value of 10,000 experimental units/ml (EU/ml). After incubation, 1 μg/ml of OVA coupled to digoxigenin (DIG), which was prepared from a kit containing DIG-3-*o*-methylcarbonyl-ε-aminocaproic acid-N-hydroxy-

succinimide-ester (Roche Diagnostics, Basel, Switzerland) in ELISA buffer was added for 1.5 h, followed by incubation with anti-DIG-Fab fragments coupled to horseradish peroxidase (Roche Diagnostics) 1:500 diluted in ELISA buffer for 1 h. Color development was performed with *o*-phenylenediamine-dichloride substrate (0.4 mg/ml, Sigma) and 4 mM H_2O_2 in PBS and stopped by adding 4 M H_2SO_4. The optical density was read at 492 nm, using a Benchmark microplate reader (Bio-Rad Laboratories Hercules, CA). The detection limit of the ELISA was 0.5 EU/ml IgE.

Analysis of the cellular composition in the BALF

Directly after bleeding of the mice, broncho-alveolar lavage was performed. Briefly, the airways were lavaged 5 times through a tracheal cannula with 1 ml aliquots of pyrogen-free saline warmed to 37°C. The recovered lavage fluid was pooled and cells herein were pelleted ($32 \times g$, 4°C, 5 min) and resuspended in 0.15 ml cold PBS. The total number of cells in the BAL fluid (BALF) was determined using a Bürker-Türk counting-chamber (Karl Hecht Assistent KG, Sondheim/Röhm, Germany). For differential BALF cell counts, cytospin preparations were made and stained with Diff-Quick (Dade A.G., Düdingen, Switzerland). Per cytospin 400 cells were counted and differentiated into mononuclear cells (monocytes, macrophages and lymphocytes), eosinophils and neutrophils by standard morphology. Statistical analysis was performed using the non-parametric Mann Whitney *U* test.

Determination of cytokine production by OVA re-stimulated lung cells in vitro

Cytokine production by antigen re-stimulated T-cells in lung tissue was determined as described previously (16). Briefly, the lungs were lavaged as described above and perfused *via* the right ventricle with 4 ml saline containing 100 U/ml heparin (Leo Pharmaceuticals, Weesp, The Netherlands) to remove any blood and intravascular leukocytes. Complete lung tissue was removed and transferred to cold sterile PBS. Lungs were then minced and digested in 3 ml RPMI 1640 containing 2.4 mg/ml collagenase A and DNase I (grade II) (both from Roche Diagnostics) for 30 min at 37°C. Collagenase activity was stopped by adding fetal calf serum (FCS). The lung tissue digest was filtered through a 70 μm nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) with 10 ml RPMI 1640 to obtain a single-cell suspension. The lung-cell suspension was washed, resuspended in culture medium (RPMI 1640 containing 10% FCS, 1% glutamax I, gentamicin [all from Life Technologies, Gaithersburg, MD] and 50 μM β-mercaptoethanol [Sigma]) and the total number of lung cells was determined using a Bürker-Türk counting-chamber. Lung cells (8×10^5 lung cells/well) were cultured in round-bottom 96-well plates (Greiner Bio-One, Kremsmuenster, Austria) in the presence of OVA (10 μg/ml) or medium only. As a positive control, cells were cultured in the presence of plate-bound rat anti-mouse CD3 mAb (clone 17A2, 50 μg/ml, coated overnight at 4°C).

Each *in vitro* stimulation was performed in triplicate. After 5 d of culture at 37°C, the supernatant was harvested, pooled per stimulation, and stored at -20°C until cytokine levels were determined by ELISA.

The IFN-γ, IL-4, and IL-5, IL-10 and IL-13 ELISAs were performed according to the manufacturer's instructions (PharMingen, San Diego, CA). The detection limits of the ELISAs were 160 pg/ml for IFN-γ, 16 pg/ml for IL-4, 32 pg/ml for IL-5 and 100 pg/ml for IL-10 and IL-13.

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). Unless stated otherwise, the differences between groups were statistically analyzed using a Student's *t*-test (two-tailed, homosedastic). Results were considered statistically different at the $p < 0.05$ level. Statistical analyses were performed using SPSS for Windows version 10.0.05 (SPSS, Chicago, IL).

RESULTS

Table 1 shows the design of two independent experiments using a novel murine model of ongoing disease. Experimental treatments were mCTLA4-IgG in Experiment A and the gold-standard dexamethasone in Experiment B. Group 5 was used to compare the effect of experimental treatment on established asthma manifestations (Group 4) to stopping with OVA aerosols while continuing with saline aerosols (hereafter referred to as the "spontaneous resolution group").

Table 1: Design of the two experiments using a novel murine model of ongoing disease.

Group	Sensitization	Challenge Aerosol (1-8)	Aerosol (9-16)	Treatment Experiment A	Experiment B
1	OVA	saline	saline	human total IgG	saline
2	OVA	saline	saline	murine CTLA4-IgG	dexamethasone
3	OVA	OVA	OVA	human total IgG	saline
4	OVA	OVA	OVA	murine CTLA4-IgG	dexamethasone
5	OVA	OVA	saline	human total IgG	saline

Mice were sensitized, challenged, and treated as described in MATERIALS AND METHODS. Each experimental group consisted of at least six animals.

Airway hyperresponsiveness in vivo is induced by the first series of OVA aerosols but disappears after the second series of aerosols

As shown previously, one of the advantages of whole-body plethysmography is that the airway responsiveness *in vivo* of experimental animals can be measured several

times during an experiment because it is performed in conscious, unrestrained animals (18). To verify that hyperresponsiveness was induced in OVA-sensitized mice by the first series of OVA aerosol challenges, airway responsiveness to aerosolized methacholine was measured 24 h in each of the two independent experiments after the first series of aerosols, just before treatment and the second series of aerosols. A dose-response curve (DRC) of doubling concentrations methacholine was performed in each mouse. The DRC to methacholine of OVA-challenged animals was significantly different ($p < 0.01$, Experiment A and $p < 0.01$, Experiment B) from those of saline-challenged mice. Figure 1A shows the responses to the methacholine DRC of saline- and OVA-challenged animals from Experiment A.

To determine if mCTLA4-IgG or dexamethasone could reverse established hyperresponsiveness, airway responsiveness was measured again 24 h after the second series of aerosols in Experiments A and B. However, in control-treated groups, OVA challenge did not result in hyperresponsiveness to methacholine compared with saline challenge; Penh values of OVA-challenged mice were just as low as those from saline-challenged animals at all concentrations of the methacholine DRC in both experiments. In Figure 1B, the maximal responses to 50 mg/ml methacholine of Experiment A are shown. Hence, an effect of mCTLA4-IgG or dexamethasone on established, OVA-induced airway hyperresponsiveness could not be determined in this model of ongoing disease.

So, OVA-challenged mice show airway hyperresponsiveness *in vivo* compared with saline-challenged animals after the first series of aerosols, but this nonspecific hyperresponsiveness disappears after the second series of aerosols.

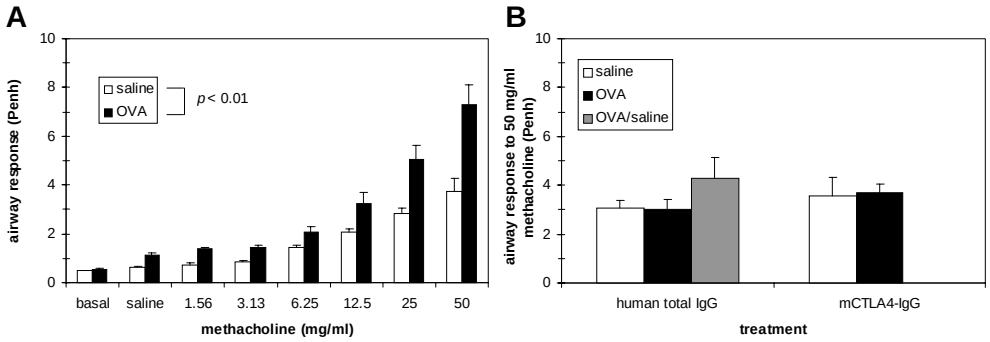

Figure 1: Airway hyperresponsiveness is induced by the first series of OVA aerosols, but has disappeared after the second series of aerosols. Airway responsiveness to aerosolized methacholine was measured in conscious, unrestrained mice 24 h after the first (A) and second (B) series of aerosols. BALB/c mice were OVA-sensitized and challenged with either eight saline aerosols ($n = 16$; *open bars*) or eight OVA aerosols ($n = 24$; *closed bars*) (A). Subsequently, mice were challenged with eight saline aerosols (*open bars* and *shaded bars*) or eight additional OVA aerosols (*closed bars*). During this second series of aerosols, mice received control treatment (human total IgG) or mCTLA4-IgG (B). Values are expressed as mean ± SEM ($n = 8$ per group). Results of Experiment A, representative for both experiments, are shown.

CTLA4-IgG inhibits the up-regulation of serum OVA-specific IgE in the ongoing murine model of airway inflammation

In Experiments A and B, which evaluated mCTLA4-IgG and dexamethasone as experimental treatment respectively, serum levels of OVA-specific IgE were determined in all mice 24 h after the second series of aerosols.

In control-treated mice, OVA challenge induces a significant up-regulation of serum OVA-specific IgE compared with saline challenge (273% increase, $p < 0.01$ and 150% increase, $p < 0.01$; shown in Figures 2A and 2B, respectively). CTLA4-IgG-treated, OVA-challenged mice show significantly reduced levels of OVA-specific IgE compared with control-treated, OVA-challenged animals (43% inhibition, $p < 0.05$). This level of allergen-specific IgE in serum was very similar to that observed in the sera of mice from the spontaneous resolution group (Group 5, Table 1) (56% inhibition compared with control-treated, OVA-challenged mice, $p < 0.01$) (Figure 2A). In contrast, dexamethasone treatment has no effect on the OVA-induced up-regulation of serum antigen-specific IgE (Figure 2B).

So, mCTLA4-IgG is able to partially inhibit the up-regulation of serum OVA-specific IgE levels in the ongoing model of airway inflammation.

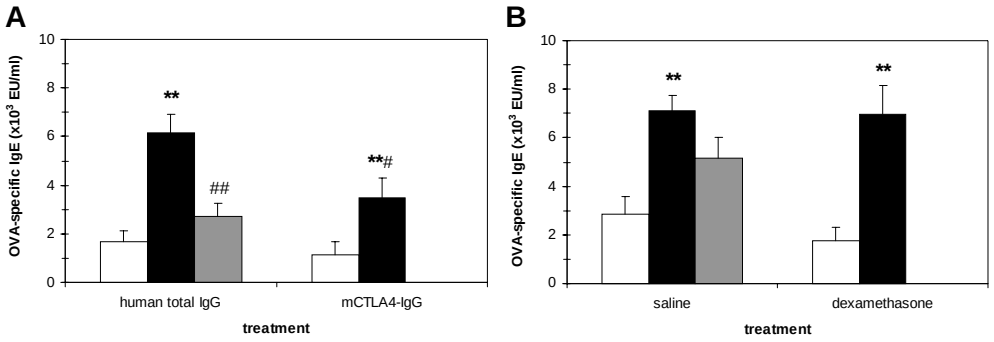

Figure 2: CTLA4-IgG inhibits the up-regulation of serum OVA-specific IgE in the ongoing murine model of airway inflammation. Serum OVA-specific IgE was determined in all mice 24 h after the second series of aerosols. BALB/c mice were OVA-sensitized and challenged with 16 saline aerosols (*open bars*), 16 OVA aerosols (*closed bars*) or 8 OVA/8 saline aerosols (*shaded bars*). During the second series of aerosols, mice received control treatment, mCTLA4-IgG (A) or dexamethasone (B). Values are expressed as mean ± SEM (n = 6-8 per group). ** $p < 0.01$, as compared with saline-challenged animals of the same treatment group. # $p < 0.05$, ## $p < 0.01$, as compared with control-treated, OVA-challenged animals.

CTLA4-IgG and dexamethasone inhibit the number of eosinophils in the BALF in the ongoing murine model of airway inflammation

The number of cells in the BALF was used as a measure for the infiltration of cells in the airways and determined in all mice 24 h after the second series of aerosols. In the lavage fluid of saline-challenged mice no (or sometimes a few) eosinophils can be observed. However, OVA challenge results in a significant ($p < 0.01$) increase in

the number of eosinophils compared with saline challenge of the same treatment group in each of the two independent experiments (Figure 3). Both mCTLA4-IgG- or dexamethasone-treated, OVA-challenged mice show significantly reduced numbers of eosinophils in the BALF compared with control-treated, OVA-challenged mice (83% inhibition, $p < 0.01$ and 97% inhibition, $p < 0.01$; shown in Figures 3A and 3B, respectively). In mice from the spontaneous resolution group, a similar reduction compared with control-treated, OVA-challenged mice was observed (82% inhibition, $p < 0.01$ and 90% inhibition, $p = 0.052$; shown in Figures 3A and 3B, respectively).

So, both mCTLA4-IgG and dexamethasone are able to inhibit the number of eosinophils in the BALF in the ongoing model of airway inflammation.

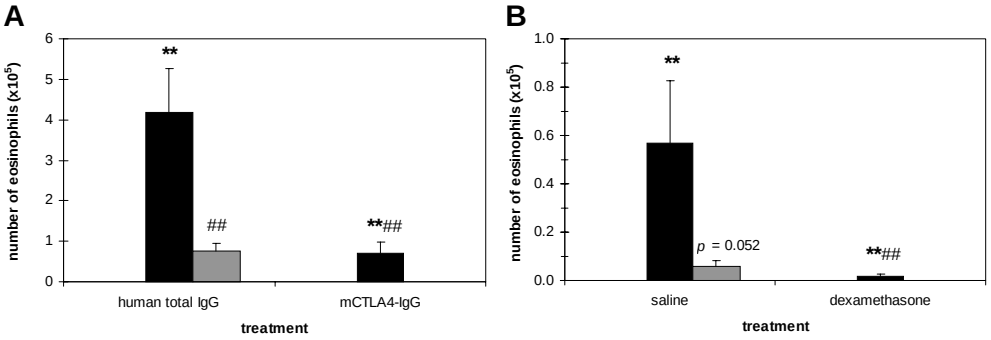

Figure 3: CTLA4-IgG and dexamethasone inhibit the number of eosinophils in the BALF in the ongoing murine model of airway inflammation. The number of eosinophils in the BALF in all mice was determined 24 h after the second series of aerosols. BALB/c mice were OVA-sensitized and challenged with 16 saline aerosols (*open bars*), 16 OVA aerosols (*closed bars*) or 8 OVA/8 saline aerosols (*shaded bars*). During the second series of aerosols, mice received control treatment, mCTLA4-IgG (A) or dexamethasone (B). Values are expressed as mean ± SEM (n = 6-8 per group). ** $p < 0.01$, as compared with saline-challenged animals of the same treatment group. ## $p < 0.01$, as compared with control-treated, OVA-challenged animals.

In each of the two independent experiments, no significant differences in the absolute number of neutrophils were observed in the BALF between the experimental groups (data not shown). In Experiment A, the absolute numbers of mononuclear cells (monocytes, macrophages and lymphocytes) were not significantly different between the experimental groups. In Experiment B, dexamethasone treatment of OVA-challenged animals resulted in a reduction in the number of mononuclear cells compared with control-treated, OVA-challenged animals (32% inhibition, $p < 0.05$).

CTLA4-IgG inhibits lung cell IL-5 production after OVA re-stimulation in vitro in the ongoing murine model of airway inflammation

A single-cell suspension of complete lung tissue of each mouse was prepared 24 h after the second series of aerosols. To determine T-cell responses upon antigenic re-stimulation, lung cells were cultured for 5 d in the presence of OVA. In agreement

with previous studies from our laboratory (16), no IFN-γ or IL-4 could be detected in the supernatant of these total lung-cell cultures after 5 d. In addition, we could not detect IL-10 and IL-13 in the supernatant of these OVA re-stimulated lung-cell cultures in this study. Lung-cell cultures derived from control-treated, OVA-challenged mice show significantly increased levels of IL-5 after re-stimulation with OVA *in vitro* compared with cultures from control-treated, saline-challenged animals (269 % increase, $p < 0.01$ and 121% increase, $p < 0.05$; shown in Figures 4A and 4B respectively). Allergen-stimulated lung-cell cultures derived from mCTLA4-IgG-treated, OVA-challenged mice show significantly reduced levels of IL-5 in the supernatant compared with cultures from control-treated, OVA-challenged animals (48% inhibition, $p < 0.05$). This degree of inhibition was similar to that observed in the cultures from mice of the spontaneous resolution group (48% inhibition, $p < 0.05$) (Figure 4A).

Dexamethasone treatment of OVA-challenged mice slightly decreased the IL-5 production by lung tissue cells after re-stimulation with OVA *in vitro*, compared with lung cells from control-treated, OVA-challenged animals (Figure 4B).

Polyclonal stimulation of T-cells with anti-CD3 mAb resulted in high levels of IFN-γ, IL-4, IL5, IL-10 and IL-13 in the supernatant of lung-cell cultures derived from all experimental groups, indicating that there was no intrinsic T-cell defect to produce any of these cytokines (data not shown).

So, mCTLA4-IgG is able to inhibit the IL-5 production by lung-cell cultures after *in vitro* re-stimulation with allergen in the ongoing model of airway inflammation.

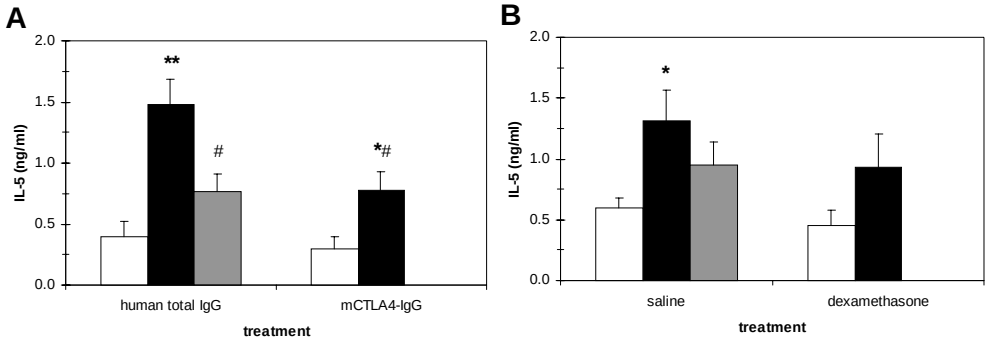

Figure 4: CTLA4-IgG inhibits lung-cell IL-5 production after OVA re-stimulation *in vitro* in the ongoing murine model of airway inflammation. Total lung-cell cultures prepared from each mouse 24 h after the second series of aerosols were cultured for 5 d in the presence of OVA. BALB/c mice were OVA-sensitized and challenged with 16 saline aerosols (*open bars*), 16 OVA aerosols (*closed bars*) or 8 OVA/8 saline aerosols (*shaded bars*). During the second series of aerosols, mice received control treatment, mCTLA4-IgG (A) or dexamethasone (B). Values are expressed as mean ± SEM (n = 11-14 per group) averaged from two independent experiments (A) or as mean ± SEM (n = 6-8 per group) (B). * $p < 0.05$. ** $p < 0.01$, as compared with saline-challenged animals of the same treatment group. # $p < 0.05$, as compared with control-treated, OVA-challenged animals.

In a more "severe" ongoing murine model, the effects of mCTLA4-IgG are less pronounced

The experiments described above are performed in a relatively mild model of airway inflammation; that is, animals are sensitized to OVA without adjuvant and challenged with low doses of OVA. Most research groups, however, use a protocol in which animals are allergen-sensitized in the presence of the Th2-dominant response-inducing alum as adjuvant and challenged with high doses of allergen. This protocol results in a model with stronger responses and appears to represent a more "severe" type of an inflammatory response. Whereas the extent of airway hyperresponsiveness is similar, ~ 10 times as much serum OVA-specific IgE and at least twice as many eosinophils in BALF can be observed after a first series of OVA aerosols when using this protocol, compared with the mild protocol (D.T. Deurloo, unpublished observations). Interestingly, it has been shown that the various models respond differently to modulation (19, 20). Therefore, we wanted to evaluate the effects of mCTLA4-IgG and dexamethasone in a more "severe" ongoing model as well (adapted from Refs. 17 and 21). Table 2 shows the design of this experiment.

Table 2: Experimental design to determine the effects of mCTLA4-IgG and dexamethasone in a more "severe" ongoing model.

Group	Sensitization	Challenge Aerosol (1-3)	Aerosol (4-6)	Treatment
1	OVA/alum	saline	saline	human total IgG
2	OVA/alum	OVA	OVA	human total IgG
3	OVA/alum	OVA	OVA	murine CTLA4-IgG
4	OVA/alum	OVA	OVA	dexamethasone
5	OVA/alum	OVA	saline	human total IgG

Mice were sensitized, challenged, and treated as described in MATERIALS AND METHODS. Each experimental group consisted of at least six animals.

In the more "severe" ongoing model, airway responsiveness to methacholine was measured in all mice 24 h after the second series of aerosols. In contrast to the milder ongoing model of airway inflammation, OVA challenge induces airway hyperresponsiveness to methacholine compared with saline challenge not only after a first series of aerosols (D.T. Deurloo, unpublished observations), but also after two series of aerosols in control-treated mice ($p < 0.05$) (Figure 5A). CTLA4-IgG- or dexamethasone-treated, OVA-challenged mice show reduced nonspecific responsiveness at the higher concentrations of methacholine, but the complete DRCs to methacholine are not significant different compared with the DRC of control-treated, OVA-challenged mice. Also, the mice from the spontaneous resolution group (Group 5, Table 2) showed no significantly reduced airway responsiveness compared with control-treated, OVA-challenged mice (Figure 5A).

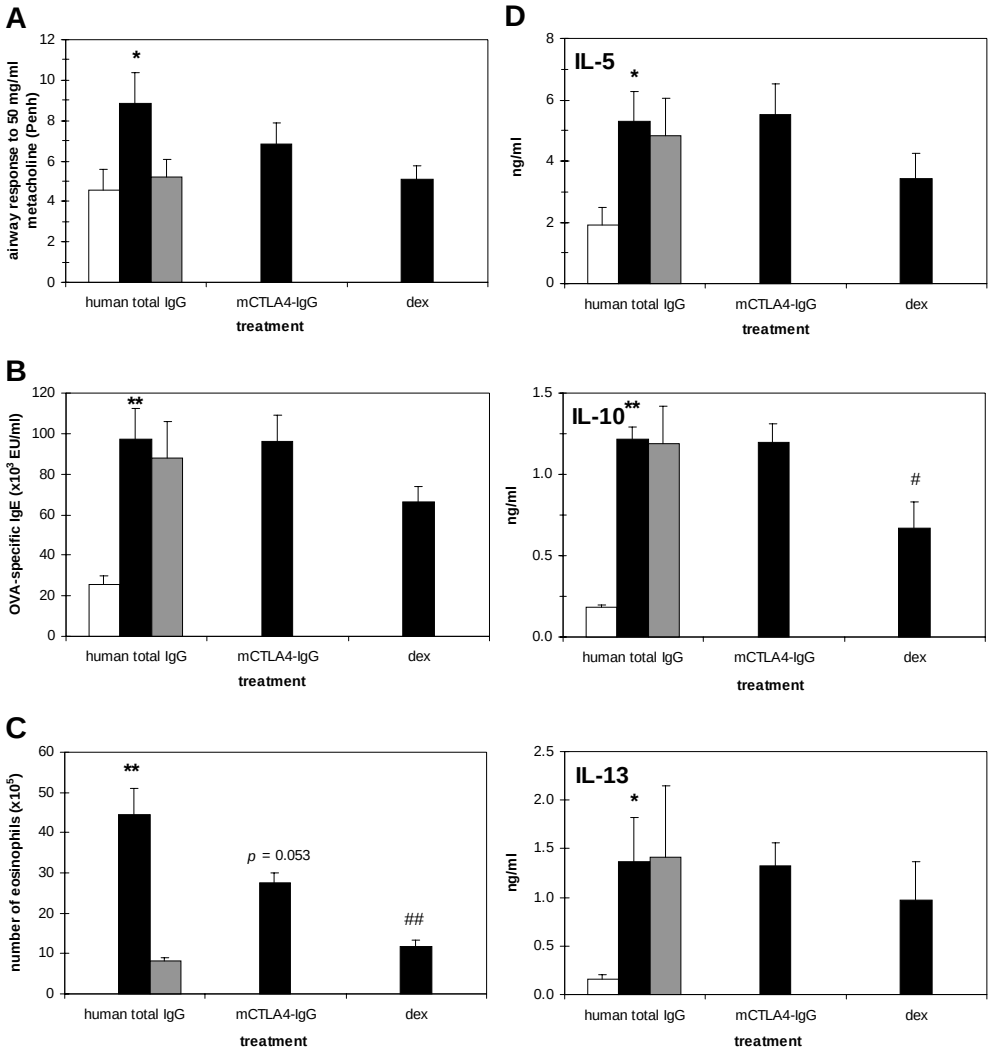

Figure 5: The effects of mCTLA4-IgG and dexamethasone in a more "severe" ongoing murine model. BALB/c mice were OVA-sensitized and challenged with six saline aerosols (*open bars*), six OVA aerosols (*closed bars*) or three OVA/three saline aerosols (*shaded bars*). Mice received control treatment (human total IgG), mCTLA4-IgG or dexamethasone (dex). Treatment started just before the second series of aerosols. Airway responsiveness to aerosolized methacholine was measured in conscious, unrestrained mice 24 h after the second series of aerosols. The response to the highest concentration (50 mg/ml) of the methacholine dose-response curve is depicted (A). Serum OVA-specific IgE (B), the number of eosinophils in the BALF (C) and cytokine production by OVA re-stimulated lung cells *in vitro* (D) was determined in all mice 24 h after the second series of aerosols. V alues are expressed as mean ± SEM (n = 6-8 per group). * $p < 0.05$, ** $p < 0.01$, as compared with saline-challenged animals. # $p < 0.05$, ## $p < 0.01$, as compared with control-treated, OVA-challenged animals.

Figure 5B shows that, in comparison with the milder model of ongoing disease, much higher serum levels of OVA-specific IgE can be observed in all experimental groups of the more "severe" ongoing model 24 h after the second series of aerosols (compare Figure 5B with Figure 2). In control-treated mice, OVA challenge induces a significant up-regulation of serum OVA-specific IgE compared with saline challenge (280% increase, $p < 0.01$). Both mCTLA4-IgG and dexamethasone have no effect on the OVA-induced up-regulation of specific IgE levels. Notably, the mice from the spontaneous resolution group show no significantly different levels of serum IgE compared with control-treated, OVA-challenged mice (Figure 5B).

Also in the more "severe" ongoing model, no eosinophils are found in the BALF of saline-challenged animals 24 h after the second series of aerosols. In control-treated mice, OVA challenge induces a significant ($p < 0.01$) increase in the number of eosinophils in the BALF compared with saline challenge (Figure 5C). The absolute number of eosinophils observed in the BALF of OVA-challenged mice in the more "severe" ongoing model is enormous compared with the number of eosinophils in the BALF recovered from OVA-challenged mice in the milder ongoing model of allergic inflammation (compare Figure 5C with Figure 3). CTLA4-IgG-treated, OVA-challenged mice have reduced numbers of eosinophils compared with control-treated, OVA-challenged animals, but this suppression does not reach the level of significance (40% inhibition, $p = 0.053$). In contrast, dexamethasone-treated, OVA-challenged mice demonstrate significantly reduced numbers of eosinophils compared with control-treated, OVA-challenged animals (73% inhibition, $p < 0.01$). In mice from the spontaneous resolution group, a similar reduction compared with control-treated, OVA-challenged mice was observed (82% inhibition, $p < 0.01$) (Figure 5C).

No significant differences in the absolute number of neutrophils were observed in the BALF between the experimental groups (data not shown). In control-treated mice, OVA challenge induces a significant increase in the number of BALF mononuclear cells (201% increase, $p < 0.01$) compared with saline challenge. Both in the spontaneous resolution group and in the group of dexamethasone-treated, OVA-challenged animals, a significant reduction in the absolute number of mononuclear cells was observed compared with control-treated, OVA-challenged animals (36% inhibition, $p < 0.05$ and 49% inhibition, $p < 0.01$).

Also in this experiment, we prepared a single-cell suspension of complete lung tissue of each mouse 24 h after the second series of aerosols to re-stimulate lung cells *in vitro*. Similar to the lung-cell cultures from the milder ongoing model of airway inflammation, also these lung-cell cultures produced no IFN-γ or IL-4 after 5 d of culture in the presence of OVA. However, lung cells obtained from control-treated, OVA-challenged mice produced significantly more IL-5, - and in this more "severe" ongoing model also IL-10 and IL-13 - compared with control-treated, saline-challenged animals upon antigenic re-stimulation (177 % increase, $p < 0.05$; 577%

increase, $p < 0.01$; and 786% increase, $p < 0.05$, respectively) (Figure 5D). The lung-cell cultures derived from mice treated with mCTLA4-IgG and challenged with OVA produced similar levels of these cytokines after OVA re-stimulation *in vitro* as those of control-treated, OVA-challenged animals. Also in the supernatant of lung-cell cultures obtained from mice from the spontaneous resolution group, no difference in the cytokine levels was observed when compared with control-treated, OVA-challenged mice. In contrast, dexamethasone significantly inhibited IL-10 production by the lung-cell cultures (45% inhibition, $p < 0.05$), whereas IL-5 and IL-13 production was slightly reduced (Figure 5D).

Polyclonal stimulation of T-cells with anti-CD3 mAb resulted in high levels of IFN-γ, IL-4, IL5, IL-10 and IL-13 in the supernatant of lung-cell cultures derived from all experimental groups (data not shown).

So, in a more "severe" ongoing model, mCTLA4-IgG was less effective compared with the milder ongoing model of airway inflammation in that only airway hyperresponsiveness was attenuated at the higher concentrations of the methacholine DRC and the number of eosinophils in the BALF was reduced. In comparison, dexamethasone not only attenuated hyperresponsiveness at the higher concentrations of the methacholine DRC, but it also significantly inhibited BALF eosinophilia and lung IL-10 production *in vitro* together with a reduction in IL-5 production.

DISCUSSION

In the present study, we sought to determine if co-stimulatory blockade by murine CTLA4-IgG can reverse established asthma manifestations in an OVA-induced murine model of ongoing disease. CTLA4-IgG inhibited the up-regulation of OVA-specific IgE in serum, BALF eosinophilia and lung cell IL-5 production *in vitro* in a mild ongoing model of airway inflammation. Whereas airway hyperresponsiveness to methacholine was present in this ongoing model in OVA-sensitized mice after the first series of OVA aerosol challenges that induced airway inflammation, this nonspecific hyperresponsiveness had disappeared after the second series of OVA aerosols. Hence, the effect of mCTLA4-IgG on this parameter could not be determined. Importantly, mCTLA4-IgG performed better than the gold-standard dexamethasone because this corticosteroid did not inhibit serum levels of OVA-specific IgE. In a more "severe" ongoing model (adapted from Refs. 17 and 21), mCTLA4-IgG was less effective in that only the number of eosinophils in the BALF was reduced ($p = 0.053$). However, in this more "severe" ongoing model, airway hyperresponsiveness was present after the second series of OVA aerosols and attenuated by mCTLA4-IgG administration. Dexamethasone inhibited both BALF eosinophilia and cytokine production by lung lymphocytes in this more "severe" ongoing murine model.

So, we are the first to demonstrate that in a murine model of ongoing disease, co-stimulatory blockade by mCTLA4-IgG can also be effective after the onset of asthma manifestations. During the time course of this ongoing model of the disease, the types and numbers of the various allergen-specific CD4+ T-cell subsets, based on their activation status, constantly changes. After sensitization, OVA-specific memory T-cells have developed. In the two models of ongoing disease, recently activated effector T-lymphocytes are present after the first series of OVA aerosol challenges at the time of treatment. In addition, naïve T-cells might be recruited and activated during the two series of OVA aerosols. *In vitro* studies have demonstrated that memory- and previously activated effector T-cells are less dependent on CD28-mediated co-stimulation than naïve T-cells (11, 13, 14), whereas *in vivo* studies on the effectiveness of giving CTLA4-Ig after priming of immune responses have yielded conflicting results (12). An example of a successful study is the recent clinical report on the administration of CTLA4-Ig to patients with *psoriasis vulgaris* in a phase I trial that produced a dose-dependent improvement in skin lesions (22). It is possible that the effectiveness of CTLA4-Ig on established disease depends on its ability to block the development and contribution to disease of newly recruited naïve CD4+ T-cells. If this is true, especially in our mild ongoing model, the contribution of newly recruited naïve CD4+ T-cells may be considerable. Alternatively, CTLA4-Ig may be effective in an ongoing or a memory response under some conditions, determined by the type of antigen inducing the immune response, the lymphoid microenvironment or the T-cell cytokine pattern elicited during the immune response (12, 13).

We investigated the role of the CD28/B7 T-cell co-stimulatory pathway in two murine models of ongoing disease. The crucial role of CD28-mediated co-stimulation in the generation of effective T-cell responses has been well documented (5). However, two new pairs of the CD28/B7 families have been identified recently (reviewed in 23). Inducible co-stimulatory molecule (ICOS), as indicated by its name, is expressed on the surface of T-cells upon activation and binds to B7RP-1 (also known as B7h, B7-H2, GL-50, ICOSL or LICOS) present on a variety of cells. Interestingly, on the basis of many recent studies, it appears that ICOS functions primarily to induce T-cell effector function. For example, engagement of ICOS appeared to be important for the cytokine responses of recently activated effector T-cells. Although ICOS engagement might support activation of naïve T-cells, it could be more important for enhancing ongoing and/or memory responses. The other new member of the CD28 family, programmed death 1 (PD-1), like CTLA4, appears to mediate an inhibitory signal to T-cells upon binding to its ligands PD-L1 (or B7-H1) and PD-L2. In contrast to CTLA4-deficient mice, which all die at three to four wk of age, severe autoimmune symptoms are observed only in approximately half of the PD-1-deficient mice, even at 14 mo of age, suggesting that PD-1 is not the primary

inhibitory signal for T-cells (23). Still more B7 family members are being identified at the time of writing this report (24, 25). A detailed understanding of the roles and interplay of these T-cell co-stimulatory pathways might result in new therapeutic possibilities for the manipulation of T-cell-mediated diseases (*e.g.*, a treatment consisting of CTLA4-Ig in combination with an agent blocking other co-stimulatory pathway[s]).

Blocking co-stimulation might not only result in the suppression of the immune response but in some cases might induce antigen-specific tolerance. When T-cells receive an antigen-specific signal through the TCR in the absence of co-stimulatory signals, this results in non-responsiveness upon subsequent optimal T-cell activation, according to the classical model of T-cell anergy (26, 27). Alternatively, some research groups have suggested that *in vivo* administration of CTLA4-Ig might leave small amounts of B7 uncovered that bind to CTLA4 due to its higher affinity. This would imply that CTLA4-Ig administration results in the absence of CD28 ligation, whereas part of the CTLA4 molecules is ligated. CTLA4 ligation has been implicated in the induction of peripheral tolerance (28, 29). Of course, tolerance induction is very attractive in the therapy of asthmatic patients because this would mean that after a single period of treatment, patients have improved clinical status for a considerable time after cessation of therapy. Therefore, we are currently investigating whether the effects of mCTLA4-IgG are transient or long lasting in the (ongoing) murine models of allergic asthma.

Allergen-specific IgE in serum, BALF eosinophilia and IL-5 production by lung cells are very characteristic features seen in patients with asthma. These manifestations were significantly inhibited by mCTLA4-IgG in the mild ongoing murine model of airway inflammation. Because IL-5 is involved in the growth, migration and activation of eosinophils, the decreased IL-5 production by lung cells isolated from mCTLA4-IgG-treated, OVA-challenged mice correlates with the inhibition of BALF eosinophilia observed in this group. Unfortunately, we cannot draw any conclusions about the effect of mCTLA4-IgG on OVA-induced airway hyperresponsiveness because hyperresponsiveness to methacholine had disappeared in control-treated, OVA-challenged animals after the second series of aerosols. We can only speculate on the mechanism of this disappearance of airway hyperresponsiveness. One possibility is that two series of eight OVA aerosols might induce tolerance. This would mean that in our milder ongoing murine model, partial tolerance is induced, namely for nonspecific hyperresponsiveness and not for the other parameters, suggesting that the loss of airway hyperresponsiveness is probably not due to immunologic tolerance. Unless hyperresponsiveness is regulated by a different subset of (allergen-specific) CD4+ T-cell than the allergen-specific CD4+ T-cell responsible for both isotype switching of B cells to IgE and BALF eosinophilia. This T-cell most likely is also CD4+ because airway hyperresponsiveness is abolished by *in vivo* depletion of CD4+ T-cells (30).

Another explanation comes from a study by Palmans and colleagues evaluating the effect of prolonged allergen exposure on airway function and structure in rats. Although airway hyperresponsiveness to aerosolized carbachol and increased total- and inner airway wall area were present after 2 wk of OVA exposure in sensitized rats, these features were absent after 12 wk of OVA exposure, whereas the influx of eosinophils into and around the airways persisted, as did IgE synthesis. The authors suggest that the loss of hyperresponsiveness is related to the development of compensatory mechanisms, the loss of which could contribute to the persistent airway hyperresponsiveness in human asthma (31). However, we wonder how likely these compensatory mechanisms apply to our mild ongoing model because both the time span of antigen exposures and the dose of antigen are much smaller. Future studies might clarify this issue.

In the more "severe" ongoing model, mCTLA4-IgG is less effective compared with the milder ongoing model. The levels of circulating OVA-specific IgE and lung IL-5 production *in vitro* are not inhibited and the number of eosinophils in the BALF is reduced to a smaller extent compared with the milder model of airway inflammation. However, nonspecific airway hyperresponsiveness is present after the second series of OVA aerosols in this more "severe" ongoing model and mCTLA4-IgG attenuated OVA-induced airway hyperresponsiveness (Figure 5A). The stronger responses of this ongoing model compared with the mild ongoing model of airway inflammation (compare Figure 5 with Figures 2-4) -hence the designation more "severe" - might be more difficult to inhibit. After induction of asthma manifestations by a first series of OVA aerosols, saline challenge of mice during the second series of aerosols (spontaneous resolution group) also resulted in no or a small diminution of allergic asthma manifestations compared with control-treated, OVA-challenged animals.

Interestingly, it has been shown that the various murine models of allergic asthma induced by different sensitization and challenge protocols respond differently to modulation. For example, no airway hyperresponsiveness was developed in mast cell-deficient mice following sensitization and challenge with OVA, whereas OVA sensitization and challenge in congenic littermates did induce nonspecific hyperresponsiveness. However, OVA-sensitized, mast cell-deficient mice developed nonspecific hyperresponsiveness by increasing the frequency and antigen dose of challenge (19). These and other findings suggest that the relative contribution of allergen-specific IgE-dependent mast cell activation and BALF eosinophilia to the development of nonspecific airway hyperresponsiveness not only depends on the strain of mice used but also on the sensitization and challenge protocol (32). So, apart from the quantitative differences between the two murine models of ongoing disease, there might be qualitative differences as well, perhaps indicating differential activation of T-cell signaling pathways under the various conditions of allergen exposure.

From the results obtained from this and previous studies (18, 33), we conclude that in our mild and more "severe" (ongoing) models, neither allergen-specific IgE nor BALF eosinophilia are solely responsible for the development or maintenance of airway hyperresponsiveness. Additional evidence for the dissociation of BALF eosinophilia and nonspecific hyperresponsiveness in the more "severe" ongoing model comes from an experiment in which two different routes of administration for mCTLA4-IgG were compared. Whereas local (intranasal) administration of mCTLA4-IgG results in a diminution of hyperresponsiveness similar to systemic (intravenous) administration of mCTLA4-IgG compared with control-treated OVA-challenged animals, only systemic administration of mCTLA4-IgG resulted in reduced BALF eosinophilia (D.T. Deurloo, unpublished observation).

For optimal treatment of asthma patients, it remains to be determined which (ongoing) model best represents their clinical situation. The use of various mouse strains combined with various sensitization- and challenge protocols might be meaningful because the group of patients with asthma is very heterogeneous, both genetically and in the way they are exposed to environmental allergens. As an advantageous feature of the mild (ongoing) model, it can be said, however, that the percentage of eosinophils found in the BALF is very similar to that observed in the lavage fluid of mild to severe asthmatics (10-20%, 24 h after allergen provocation [34]).

The gold-standard dexamethasone performed equally well as mCTLA4-IgG in the mild ongoing model of airway inflammation, except that circulating OVA-specific IgE levels were not inhibited. This observation on allergen-specific IgE levels is in agreement with previous studies both in humans and mice (35, 36). In the more "severe" ongoing model, however, dexamethasone treatment of OVA-challenged mice was more effective in comparison with mCTLA4-IgG administration. If we extrapolate the results from this study to the human situation, these data imply that patients with mild disease can benefit from specific treatment with CTLA4-Ig, whereas the more generally suppressive drug dexamethasone, perhaps in combination with CTLA4-Ig, is required for patients suffering from more severe disease.

In summary, we described the effects of mCTLA4-IgG after induction of asthma manifestations in two murine models of ongoing disease, which differed in severity of the disease. In a mild ongoing model of allergic airway inflammation, mCTLA4-IgG was very effective, whereas in a more "severe" ongoing model, its effects were less pronounced. We conclude from this study that CTLA4-Ig might be an effective alternative therapy for established allergic asthma, especially in situations of mild disease. Future experimental studies investigating the long-term effects of mCTLA4-IgG in (ongoing) murine models of allergic asthma will further clarify its therapeutic potential.

ACKNOWLEDGEMENTS

D.T.D. and E.C.A.M.v.E. are supported by Research Grant 3.2.97.09 from The Netherlands Asthma Foundation. Murine CTLA4-IgG was kindly provided by Dr. P. Jardieu (Immunology, Genentech, South San Francisco, CA)

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Chapter 4

Blockade of ICOS fails to reverse established asthma manifestations in an ongoing murine model

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ABSTRACT

Asthmatic patients usually start therapy after the development of airway symptoms, while exposure to environmental allergen mostly continues. Current data suggest that inducible T-cell co-stimulator (ICOS) has a more prominent role in the effector phase than in the initiation of Th2-dominated allergic inflammatory responses. In the present study, we compared the effects of ICOS blockade during the development of asthma-like symptoms in mice (induction model) with the effects of ICOS blockade after the development of airway symptoms when antigen exposures were continued (ongoing model). For the induction model, BALB/c mice were sensitized to ovalbumin (OVA) and challenged with three OVA aerosols. Treatment of mice with ICOS-Fc throughout the aerosol challenge period significantly reduced the number of inflammatory cells in the broncho-alveolar lavage fluid compared with control-treated animals but airway hyperresponsiveness *in vivo* and serum OVA-specific IgE levels were unaltered. In the ongoing model, OVA-sensitized mice received ICOS-Fc solely during the period of the fourth to sixth aerosol, after development of asthma manifestations. Administration of ICOS-Fc had no effect on any of the final asthma manifestations. Likewise, ICOS-Fc did not inhibit IL-5 production by primed OVA-specific lung T-cells *ex vivo*. So, in this ongoing murine model, ICOS appears to play no role in the regulation of Th2 effector cell function in the lung.

INTRODUCTION

Allergic asthma is a complex inflammatory disease of the airways characterized by reversible airflow obstruction, persistent airway hyperresponsiveness, chronic eosinophilic airway inflammation and airway remodeling (1). Allergen-specific T helper (Th) type 2 cells play a pivotal role in both the initiation and progression of allergic asthma by orchestrating the inflammatory response. Th2 cells are characterized by the array of effector cytokines they secrete, such as IL-4, IL-5, IL-9, IL-10 and IL-13, which are optimized to combat parasite infections (2). However, Th2 cells are developed undesirably in allergic disorders such as asthma, their cytokines causing airway symptoms either directly or mediated by other immune cells and their mediators (3). In animal models of allergic asthma, various experimental treatments have therefore been explored that target the allergen-specific T-cell, including blockade of their co-stimulatory requirements (4, 5).

For optimal activation, CD4+ T-cells require nonspecific co-stimulatory signals in addition to the antigen-specific signal conferred by the T-cell receptor (TCR) (6-8). CD28 is the best-characterized T-cell co-stimulatory receptor, constitutively present on the surface of naïve T-cells. Engagement of CD28 by its ligands B7-1 (CD80) or B7-2 (CD86) on antigen-presenting cells provides potent co-stimulatory signals to T-cells activated through their TCR that enhance activation and sustain the T-cell response. The second receptor for the B7 ligands, CTLA4 (CD152), is expressed on activated T-cells and delivers inhibitory signals to terminate the T-cell response (9).

The critical role of CD28 signaling in the development of Th2-dominated allergic inflammatory responses in mice has been demonstrated in both gene targeting and blocking studies. No asthma-like symptoms could be induced in CD28-deficient ($CD28^{-/-}$) mice (Refs. 10-12 and chapter 2) or B7-1/B7-$2^{-/-}$ mice (Ref 13 and chapter 2). Administration of CTLA4-Ig or monoclonal antibodies (mAbs) against the B7 molecules at the time of antigen sensitization or challenge of wild-type mice greatly reduced the allergen-induced manifestations of asthma (14-18).

In contrast to these experimental studies, asthmatic patients start therapy after the development of airway symptoms, while exposure to environmental allergen mostly continues. Studies *in vitro* have demonstrated that effector and memory T-cells are less dependent on co-stimulation in comparison with naïve T-cells (19-21), whereas studies *in vivo* on the effectiveness of giving CTLA4-Ig after priming of immune responses have yielded conflicting results (22). In an ovalbumin (OVA)-induced murine model of ongoing disease in which treatment started after the development of asthma-like symptoms and antigen exposures were continued, CTLA4-IgG was still effective (23). However, airway eosinophilia and the up-regulation of OVA-specific IgE levels could only be partially inhibited. In this ongoing

murine model of allergic asthma, the partial inhibition by CTLA4-IgG may be due to the predominance of antigen-experienced (memory and effectors) over newly recruited naïve OVA-specific T-cells at the time of treatment. These antigen-experienced T-cells may be regulated by other co-stimulatory molecules.

A possible candidate is the recently discovered CD28 family member inducible co-stimulator (ICOS) that binds its own B7 family member, B7 related protein 1 (B7RP-1) (also known as B7h, B7-H2, GL50 or ICOSL or LICOS) (reviewed in 24, 25). ICOS is induced rapidly on T-cells after TCR engagement and is retained on many memory T-cells, suggesting that ICOS provides co-stimulatory signals to activated T-cells. Indeed, during the initial activation of naïve T-cells, the effects of ICOS on T-cell proliferation and IL-2 production are modest in comparison to those of CD28. Signals through ICOS seem to be more important for the regulation of cytokine production by effector T-cells.

$ICOS^{-/-}$ mice were susceptible to the influx of inflammatory cells in the broncho-alveolar lavage fluid (BALF) induced by airway challenge in primed mice (26). In blocking studies, the ICOS:B7RP-1 pathway was interrupted during antigen-sensitization and/or airway challenge (27, 28) or solely during the final aerosol challenge (29). Collectively, these blocking studies suggest that ICOS co-stimulation may contribute more to the effector phase than to the initiation of Th2-dominated allergic inflammatory responses (26-29).

In the present study, we compared the effects of ICOS blockade by ICOS-Fc during the development of asthma-like symptoms in mice (induction model) with the effects of ICOS-Fc administration after the development of airway symptoms when antigen exposures were continued (ongoing model) (23). In both OVA-induced models, airway hyperresponsiveness to methacholine *in vivo*, OVA-specific IgE levels in serum and the cellular composition of the BALF was determined at 24 h after the last aerosol. In agreement with an earlier study (27), administration of ICOS-Fc significantly inhibited the number of inflammatory cells in the BALF, but had no effect on airway hyperresponsiveness *in vivo* and serum OVA-specific IgE levels. However, in the ongoing model, administration of ICOS-Fc failed to reduce any of the final asthma-like symptoms. Likewise, ICOS-Fc did not inhibit lung cell IL-5 production upon OVA re-stimulation *in vitro*. So, in this ongoing murine model, ICOS appears to play no role in the regulation of Th2 effector cell function in the lung.

MATERIALS AND METHODS

Animals

Specific pathogen-free male BALB/c mice were obtained from Charles River (Maastricht, The Netherlands). Six wk old mice were used for our studies. Animal

care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments.

Determination of the ability of human ICOS-Fc to bind murine B7RP-1
The ability of human ICOS fused to the Fc region of human IgG (hICOS-Fc; produced in the laboratory of Dr. A.J. Coyle, Millenium Pharmaceuticals, Cambridge, MA, USA) to bind murine B7RP-1 was determined by flow cytometric analysis. CHO cells stably transfected with cDNA encoding murine B7RP-1 (kindly provided by Dr. C.P.M. Broeren, Department of Immunology, Faculty of Veterinary Medicine, Utrecht University) or non-transfected CHO cells (10^5 cells/well) were incubated in a 96 well round-bottomed plates (Greiner Bio-One, Kremsmuenster, Austria) with FACS buffer (PBS, 1% fetal calf serum [FCS], 0.1% NaN_3) containing 1:500 diluted supernatant of the hybridoma of the 2.4G2, IgG2b mAb (rat anti mouse FcγII/III receptor). After blocking, cells were incubated with 10 μg/ml human IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or hICOS-Fc. Hereafter, cells were incubated with 1 μg/ml goat $F(ab')_2$ anti-human IgG-γ-FITC (Protos Immunosearch, Burlingame, CA, USA). All incubations were performed for 20 min at 4°C in the dark. Cells were analyzed on a FACS Calibur using Cell Quest (Becton-Dickinson, San Jose, CA, USA).

Culture of thoracic lymph node- and lung cells
Mice (n = 4) were sensitized and challenged as described below for the experiment in the induction model of allergic asthma. At 24 h after the last OVA aerosol, lungs were lavaged as described below. First, lung-draining lymph nodes were collected from the thorax and transferred to cold sterile PBS. Thoracic lymph nodes (TLNs) from four mice were pooled; this experiment was performed in duplicate. Next, lungs were perfused *via* the right heart ventricle with 4 ml saline at 37°C containing 100 U/ml heparin (Leo Pharmaceuticals, Weesp, The Netherlands) to remove any blood and intravascular leukocytes. Complete lung tissue was removed and transferred to cold sterile PBS and subsequently cultured from each mouse individually.

TLNs were filtered through a 70-μm nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA) with 5 ml RPMI 1640 to obtain a single-cell suspension. Lungs were minced and digested in 3 ml RPMI 1640 containing 2.4 mg/ml collagenase A and 1.0 mg/ml DNase I (grade II) (both from Roche Diagnostics, Basel, Switzerland) for 30 min at 37°C. Enzyme activity was stopped by adding 1 ml FCS. The lung tissue digest was filtered with 10 ml RPMI 1640 to obtain a single-cell suspension. The TLN- and lung-cell suspensions were washed, resuspended in culture medium (RPMI 1640 supplemented with 10% FCS, 1% glutamax I and gentamicin [both from Life Technologies, Gaithersburg, MD, USA]

and 50 μM β-mercaptoethanol [Sigma]), and the total number of cells were determined using a Bürker-Türk counting-chamber.

Cells (2×10^5 TLN cells/well, 8×10^5 lung cells/well) were cultured in 96-well round-bottomed plates (Greiner Bio-One) in the presence of OVA (chicken egg albumin, crude grade V, Sigma, St. Louis, MO, USA). (10 μg/ml) and various concentrations of hICOS-Fc or human total IgG (ranging from 0-50 μg/ml). In addition, cells were cultured with medium only as negative control. After 5 d of culture at 37°C in 5% CO_2, the supernatant was harvested, and stored at -20°C until determination of cytokine levels by ELISA.

Antigen sensitization and challenge and experimental treatment protocol
All mice were sensitized to OVA. Systemic sensitization was performed by two intraperitoneal (i.p.) injections of 10 μg OVA adsorbed onto 2.25 mg aluminum hydroxide (alum; ImjectAlum, Pierce, Rockford, IL, USA) in 0.1 ml pyrogen-free saline on day 0 and 7.

For the experiment in the induction model of allergic asthma, mice were challenged three times by inhalation of an OVA aerosol for 20 min on day 28, 31 and 34. Throughout the antigen challenge period (day 28-34), mice received 70 μg human total IgG as control treatment or 70 μg hICOS-Fc as experimental treatment dissolved in 0.2 ml pyrogen-free saline (one i.p. injection per day). This dosage regimen was designed on the basis of successful studies with ICOS-Ig described in literature (27-30).

For the experiment in the ongoing murine model of allergic asthma, mice received six saline aerosols, six OVA aerosols, or three OVA aerosols followed by three saline aerosols ("spontaneous resolution group") for 20 min on day 27, 30, 33, 36, 39 and 42 (*see* Table 1). Throughout the period of the fourth to sixth aerosol challenge (day 36-42), mice received 70 μg control treatment or 70 μg hICOS-Fc in 0.2 ml pyrogen-free saline (one i.p. injection per day) as indicated in Table 1. On days that treatment coincided with an aerosol challenge, treatment was administered 30 min before aerosol challenge.

The aerosols were generated in a plexiglass exposure chamber (5 liter) by nebulizing an OVA solution (10 mg/ml in pyrogen-free saline) or saline using a Pari LC Star nebulizer (PARI Respiratory Equipment, Richmond, VA, USA; particle size 2.5–3.1 μm) driven by compressed air at a flow rate of 6 liters/min.

Human total IgG was purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA) and dissolved in pyrogen-free saline. This solution was treated with 10% (v/v) polymyxin B-agarose (Sigma; washed twice with saline) for 1 h at 4°C to remove lipopolysaccharide. After incubation, agarose beads were removed by centrifugation, and the supernatant was sterilized.

Measurement of airway responsiveness in vivo

Airway responsiveness was measured in conscious, unrestrained mice on day 22 and 35 (6 d before the OVA aerosol challenge period and at 24 h after the last OVA aerosol challenge, respectively [experiment in the induction model of allergic asthma]) or on day 43 only (at 24 h after the last aerosol challenge [experiment in the ongoing model of allergic asthma]), by recording respiratory pressure curves in response to inhaled nebulized methacholine (methacholine (acetyl-β-methylcholine chloride; Sigma) using barometric whole-body plethysmography (Buxco; EMKA Technologies, Paris, France). Airway responses were expressed in enhanced pause (Penh), an index of airway obstruction as described in detail previously (31). Briefly, mice were placed in a whole-body chamber and pressure differences between this chamber and a reference chamber were recorded. After baseline Penh values were obtained for 3 min and averaged, animals were exposed to a saline aerosol and a series of methacholine aerosols (solutions doubling in concentration, ranging from 1.56–50 mg/ml in saline). Aerosols were generated by a Pari LC Star nebulizer for 3 min and after each nebulization readings were taken for 3 min and averaged.

Determination of OVA-specific IgE levels in serum

In the experiment in the induction model of allergic asthma, at day 25 (3 d before the OVA aerosol challenge period), approximately 0.25 ml blood was recovered from mice by an incision the tail vein. After measurement of airway responsiveness *in vivo* at 24 h after the last OVA aerosol challenge on day 35 (experiment in the induction model of allergic asthma) or day 43 (experiment in the ongoing model of allergic asthma), mice were sacrificed by i.p. injection of 1 ml 10% urethane (Sigma) in sterile saline and bled by cardiac puncture. Serum was prepared from blood samples and stored at -20°C until determination of OVA-specific IgE levels by ELISA. Briefly, maxisorp 96-wells flat-bottomed microtiter plates (Nunc A/S, Roskilde, Denmark) were coated overnight at 4°C with 1 μg/ml rat anti-mouse IgE mAb (clone R35-72, PharMingen, San Diego, CA, USA) diluted in phosphate-buffered saline (PBS). The next day, the ELISA was performed at room temperature using ELISA buffer (PBS containing 0.5% bovine serum albumin [BSA, Sigma], 2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.05% Tween-20 [Merck, Whitehouse Station, NJ, USA], pH 7.2) for blocking and dilution of samples, standard and reagents and PBS containing 0.05% Tween-20 for washing between incubations. After blocking of wells for 1 h, serum samples and a duplicate dilution series of an OVA-specific reference serum (starting 1:40) were added to the wells and incubated for 2 h. An OVA-specific IgE reference serum was obtained by sensitization and challenge of mice with OVA as above-described for the induction model of allergic asthma and arbitrarily assigned a value of 1,000 experimental units/ml (EU/ml). Hereafter, 1 μg/ml of OVA labeled to digoxigenin (DIG) by a DIG protein labeling kit (Roche Diagnostics) was added

for 1.5 h, followed by incubation with 1:500 diluted anti-DIG-Fab fragments coupled to horseradish peroxidase (Roche Diagnostics) for 1 h. Color development was performed with *o*-phenylenediamine-dichloride substrate (0.4 mg/ml, Sigma) and 4 mM H_2O_2 in PBS and the reaction was stopped by adding 4 M H_2SO_4. The optical density was read at 490 nm, using a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Results were analyzed with Microplate Manager PC software (Bio-Rad Laboratories). The lower detection limit of the ELISA was 4 EU/ml IgE.

Analysis of the BALF

BAL was performed immediately after bleeding of the mice by lavage of the airways through a tracheal cannula with 1 ml saline at 37°C containing 5% BSA and 2 μg/ml aprotinine (Roche Diagnostics). The recovered lavage fluid of this first ml was kept apart for determination of cytokines in the supernatant by ELISA. Subsequently, mice were lavaged four times with 1 ml aliquots of saline only at 37°C. Recovered lavage fluid of the second through fifth ml was pooled and cells (including those from the first ml) were pelleted ($387 \times g$, 4°C, 10 min), and resuspended in 0.15 ml cold PBS. The total number of cells in the BALF was determined using a Bürker-Türk counting-chamber (Karl Hecht Assistent KG, Sondheim/Röhm, Germany). For differential BALF cell counts, cytospin preparations were made ($15 \times g$, 4°C, 5 min) using a cell container (Kendro Heraues Instruments, Asheville, NC, USA), and cells were fixed and stained with Diff-Quick (Dade AG, Düdingen, Switzerland). Per cytospin, at least 200 cells were counted and differentiated into mononuclear cells (monocytes, macrophages and lymphocytes), eosinophils and neutrophils by standard morphology and staining characteristics.

Measurement of cytokines

Supernatants of the first ml BALF and TLN cell-cultures were analyzed for IFN-γ, IL-4, IL-5 and IL-10 content by sandwich ELISA using Ab pairs and standards purchased from PharMingen, according to the manufacturer's instructions. The lower detection limits of the ELISAs were 16 pg/ml for IFN-γ, 16 pg/ml for IL-4, 32 pg/ml for IL-5, and 63 pg/ml for IL-10.

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). The airway dose-response curves to methacholine were statistically analyzed by a general linear model of repeated measurements followed by *post-hoc* comparison between groups. Data were log10 transformed before analysis to equalize variances in all groups. Statistical analysis on BALF cell counts was performed using the non-parametric Mann Whitney *U* test. For all other parameters, results were statistically analyzed using a Student's

t test (two-tailed, homosedastic). Differences between groups were considered statistically significant at the $p < 0.05$ level. Statistical analyses were performed using SPSS for Windows version 10.0.5 (SPSS, Chicago, IL, USA).

RESULTS

Human ICOS-Fc binds murine B7RP-1

First, the ability of human ICOS fused to the Fc region of human IgG (hICOS-Fc) to bind murine B7RP-1 was examined by flow cytometric analysis. To this end, CHO cells stable transfected with cDNA encoding murine B7RP-1 were pre-incubated with 10 μg/ml hICOS-Fc or isotype control antibody (human IgG), followed by incubation with goat anti-human-IgG-FITC. As shown in Figure 1A, pre-incubation of cells with hICOS-Fc resulted in a shift of the fluorescence signal. Untransfected CHO cells displayed no staining upon this pre-incubation (Figure 1B).

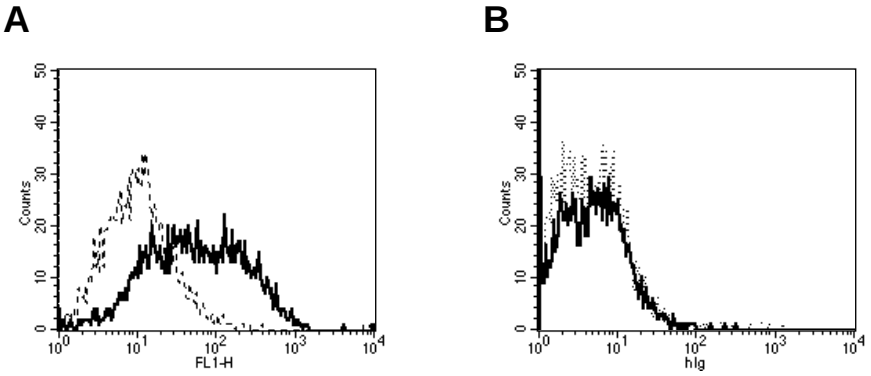

Figure 1: Human ICOS-Fc binds murine B7RP-1. CHO cells transfected with murine B7RP-1 (A) or untransfected CHO cells (B) were pre-incubated with 10 μg/ml hICOS-Fc (solid line) or isotype control antibody (human IgG, dotted line), followed by incubation with goat anti-human-IgG-FITC.

Human ICOS-Fc inhibits IL-5 production by primed murine lymphocytes derived from the thoracic lymph nodes but not those derived from the lungs in vitro

Next, the effect of hICOS-Fc on cytokine production by primed antigen-specific T-cells was determined *ex vivo*. Asthma-like symptoms were induced in BALB/c mice by systemic sensitization to OVA adsorbed to alum followed by three OVA aerosol challenges. At 24 h after the last aerosol, single-cell suspensions of the thoracic lymph nodes (TLNs) and lung tissue were prepared. Lymphocytes were re-stimulated for 5 d with 10 μg/ml OVA in the presence of various concentrations of either hICOS-Fc or human total IgG as control. As shown in Figure 2, in the presence of human total IgG, lymphocytes derived from both tissues produced IL-5 upon re-stimulation with OVA, whereas no IFN-γ could be detected in the supernatants above medium

control values (data not shown), indicative of a Th2 response. Human ICOS-Fc dose-dependently inhibited IL-5 production by TLN cells upon antigenic re-stimulation (Figure 2A). Notably, hICOS-Fc did not inhibit IL-5 production by OVA re-stimulated lung lymphocytes (Figure 2B).

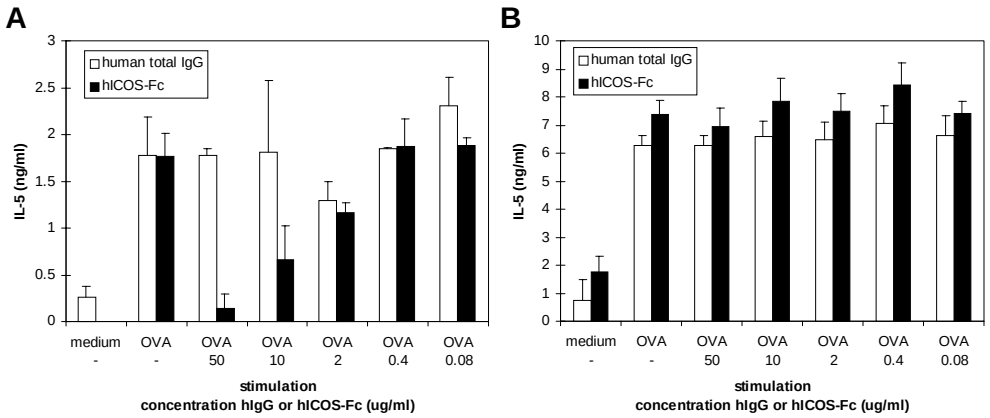

Figure 2: Human ICOS-Fc inhibits IL-5 production by primed murine lymphocytes derived from the thoracic lymph nodes but not those derived from the lungs *in vitro*. BALB/c mice ($n = 4$) were sensitized to OVA adsorbed to alum and challenged with three OVA aerosols. At 24 h after the last OVA aerosol challenge, single-cell suspensions of the thoracic lymph nodes (A; pooled from four mice; experiment performed in duplicate) and lung tissue (B; cultured from each mouse individually) were prepared. Lymphocytes were re-stimulated with 10 μg/ml OVA in the presence of various concentrations of either hICOS-Fc or human total IgG as control, and after 5 d the level of IL-5 in the supernatant of the cultures was determined.

Effects of ICOS blockade on the development of asthma-like symptoms in mice

The effects of ICOS blockade *in vivo* were first determined on the development of Th2-dominated allergic inflammatory responses. To this end, asthma-like symptoms were induced in BALB/c mice by systemic sensitization to OVA adsorbed to alum followed by three OVA aerosol challenges. Throughout the antigen challenge period, one group of animals received human total IgG as control treatment and a second group received hICOS-Fc.

Airway hyperresponsiveness in vivo

Airway responsiveness to methacholine *in vivo* was determined in conscious, unrestrained mice by barometric whole-body plethysmography and was measured well after sensitization to OVA but before the aerosol challenge period and at 24 h after the last OVA aerosol. As shown in Figure 3A, OVA-sensitized, yet untreated, BALB/c mice demonstrated a dose-dependent increase in Penh, an index of airway obstruction, in response to aerosolized methacholine. Upon OVA aerosol challenge, control-treated mice developed non-specific airway hyperresponsiveness because the complete methacholine dose-response curve (DRC) is significantly different from the DRC obtained before challenge ($p < 1 \times 10^{-6}$). Treatment of mice with hICOS-Fc

during the OVA aerosol challenge period had no effect on the development of hyperresponsiveness *in vivo*. At the higher concentrations of the methacholine DRC, the airway responses of hICOS-Fc-treated mice seemed to be even higher compared with control-treated animals. However, both the complete DRC and the airway responses to the separate concentrations of methacholine were not significantly different between the two treatment groups.

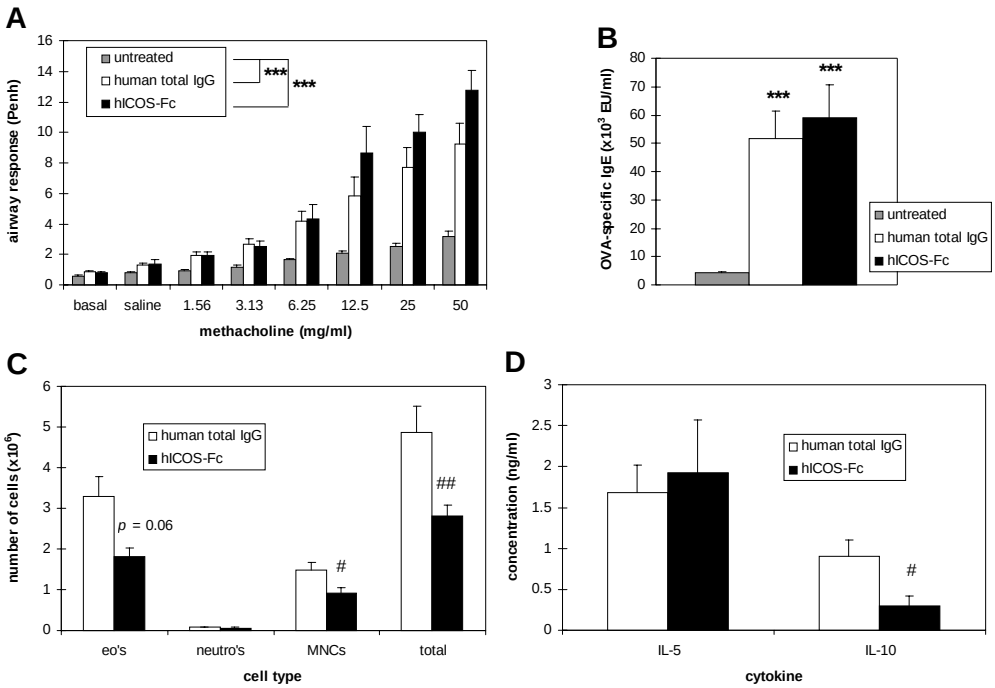

Figure 3: Effects of ICOS blockade on the development of asthma-like symptoms in mice. BALB/c mice were sensitized to OVA adsorbed to alum and challenged with three OVA aerosols. Throughout the aerosol challenge period, mice received control treatment (human total IgG, *open bars*; $n = 8$) or hICOS-Fc (*closed bars*; $n = 8$). Airway responsiveness *in vivo* to aerosolized methacholine (A) and OVA-specific IgE levels in serum (B) were first determined well after sensitization to OVA but before the OVA aerosol challenge period in yet untreated animals (*shaded bars*; $n = 16$). At 24 h after the last OVA aerosol, airway responsiveness *in vivo* (A) and OVA-specific IgE levels in serum (B) were determined again in addition to cellular compostion in the BALF (C) and the level of Th2 cytokines in the BALF (D). Values are expressed as mean ± SEM. *** $p < 0.001$, as compared with yet untreated, OVA-sensitized animals. # $p < 0.05$ and ## $p < 0.01$, as compared with control-treated, OVA-challenged animals.

OVA-specific IgE levels in serum

To determine allergen-specific IgE levels in serum, blood was withdrawn from each mouse well after sensitization to OVA but before the aerosol challenge period and at 24 h after the last OVA aerosol. In sera from naïve BALB/c mice, no OVA-specific IgE could be detected (chapter 2). After sensitization to OVA, antigen-specific IgE

was found in the sera from the yet untreated mice (4,171 ± 675 EU/ml) (Figure 3B). In control-treated mice, these levels were greatly up-regulated upon OVA aerosol challenge (51,458 ± 9,820 EU/ml, $p < 1 \times 10^{-6}$). Treatment of mice with hICOS-Fc during the OVA aerosol challenge period had no effect on this up-regulation induced by OVA challenge (59,141 ± 11,594 EU/ml).

Cellular and cytokine composition of the BALF

The number of various leukocytes in the BALF was used as a measure for the infiltration of these cells in the airways and was determined at 24 h after the last OVA aerosol challenge. In naïve or OVA-sensitized, saline-challenged mice only some mononuclear cells (monocytes, macrophages and lymphocytes) could be observed in the BALF (chapters 2, 3 and 5) (Figure 4C). In OVA-sensitized, control-treated mice, OVA aerosol challenge resulted in an influx of inflammatory cells in the BALF, consisting predominantly of eosinophils besides an increase in mononuclear cells and the appearance of a few neutrophils (Figure 3C). In the group of mice treated with hICOS-Fc during the OVA aerosol challenge period, very similar percentages of each cell type were observed compared with control-treated mice. However, the total number of cells in the BALF induced by OVA challenge was significantly inhibited in hICOS-Fc-treated mice compared with control-treated animals ($p < 0.01$) and consequently the absolute numbers of eosinophils and mononuclear cells ($p = 0.06$ and $p < 0.05$, respectively).

The influx of inflammatory cells in the BALF upon OVA aerosol challenge of OVA-sensitized, control-treated mice was accompanied by the presence of IL-5 and IL-10 (1.68 ± 0.33 ng/ml and 0.91 ± 0.20 ng/ml, respectively), but no detectable levels of IFN-γ, pointing to a Th2-dominated allergic inflammatory response (Figure 3D). Treatment of mice with hICOS-Fc during the OVA aerosol challenge period had no effect on the level of IL-5 in the BALF (1.92 ± 0.65 ng/ml) induced by OVA challenge, but resulted in a significantly reduced level of IL-10 (0.29 ± 0.13 ng/ml, $p < 0.05$) compared with control-treated animals.

So, blockade of ICOS during the allergen challenge period of OVA-sensitized mice had no effect on the development of airway hyperresponsiveness *in vivo* and the up-regulation of OVA-specific IgE levels in serum. However, the total number of cells and the level of IL-10 but not IL-5 in the BALF were significantly reduced.

Effects of ICOS blockade in an ongoing murine model of allergic asthma

Subsequently, we sought to determine the effects of ICOS blockade in an ongoing murine model of allergic asthma (*see* Table 1). Group 4 was included in the experiment to compare the effects of hICOS-Fc on established asthma manifestations with ongoing OVA exposures (Group 3) to stopping with OVA aerosols and continuing with saline aerosols (hereafter referred to as the “spontaneous resolution group”).

Table 1: Experimental design to determine the effects of ICOS blockade in an ongoing murine model of allergic asthma.

Group	Sensitization	Challenge Aerosol 1-3	Aerosol 4-6	Treatment (throughout aerosol 4-6)
1	OVA/alum	Saline	Saline	Human total IgG
2	OVA/alum	OVA	OVA	Human total IgG
3	OVA/alum	OVA	OVA	Human ICOS-human Fc
4	OVA/alum	OVA	Saline (spontaneous resolution group)	Human total IgG

Mice were sensitized, challenged, and treated as described in MATERIALS AND METHODS. Each experimental group consisted of eight animals.

Airway hyperresponsiveness in vivo

Airway responsiveness to methacholine *in vivo* was determined at 24 h after the last (sixth) aerosol challenge. In control-treated mice, OVA aerosol challenge of OVA-sensitized mice resulted in non-specific airway hyperresponsiveness because the complete methacholine DRC of this group of mice was significantly different ($p < 0.00005$) from that of saline-challenged mice (Figure 4A). Treatment of mice with hICOS-Fc after the development of asthma manifestations during ongoing OVA aerosols had no effect on the final airway hyperresponsiveness. The spontaneous resolution group showed a reduced final non-specific hyperresponsiveness *in vivo* compared with control-treated, OVA-challenged mice (Group 2), but the difference between the methacholine DRCs of these two groups of animals did not reach statistical significance ($p = 0.12$).

OVA-specific IgE levels in serum

After measurement of airway responsiveness *in vivo*, mice were sacrificed and bled by cardiac puncture to determine allergen-specific IgE levels in serum. In control-treated mice, OVA aerosol challenge of OVA-sensitized mice resulted in an up-regulation of serum OVA-specific IgE compared with saline-challenged animals (49,991 ± 6,094 EU/ml versus 28,982 ± 11,911 EU/ml, $p = 0.14$) (Figure 4B). Treatment of mice with hICOS-Fc after the development of asthma manifestations during ongoing OVA aerosols had no effect on the final levels of antigen-specific IgE in serum induced by OVA aerosol challenge (52,767 ± 9,625 EU/ml). Also the serum OVA-specific IgE levels from the spontaneous resolution group were similar to those of control-treated, OVA-challenged mice (44,435 ± 8,051 EU/ml).

Cellular composition of the BALF

After cardiac puncture, the lungs were lavaged to determine the numbers of various inflammatory cell types that had infiltrated the airway lumen. In control-treated mice, OVA aerosol challenge of OVA-sensitized mice resulted in a significant ($p < 0.0001$) increase in the number of eosinophils, mononuclear cells and hence the total number

of cells, compared with saline-challenged animals (Figure 4C). The appearance of a few neutrophils in the BALF of some animals upon OVA-challenge is too small to be seen in Figure 4C (9,842 ± 5,386). Treatment of mice with hICOS-Fc after the development of asthma manifestations during ongoing OVA aerosols slightly reduced the total number of inflammatory cells and therefore the numbers of eosinophils and mononuclear cells compared with control-treated mice, but none of these reductions did reach statistical significance ($p = 0.14$, $p = 0.28$ and $p = 0.08$, for the total number of cells, the number of eosinophils and mononuclear cells, respectively). In the spontaneous resolution group, the final number of eosinophils and consequently the total number of cells were significantly reduced compared with control-treated, OVA-challenged animals ($p < 0.0005$ for the number of eosinophils and total number of cells).

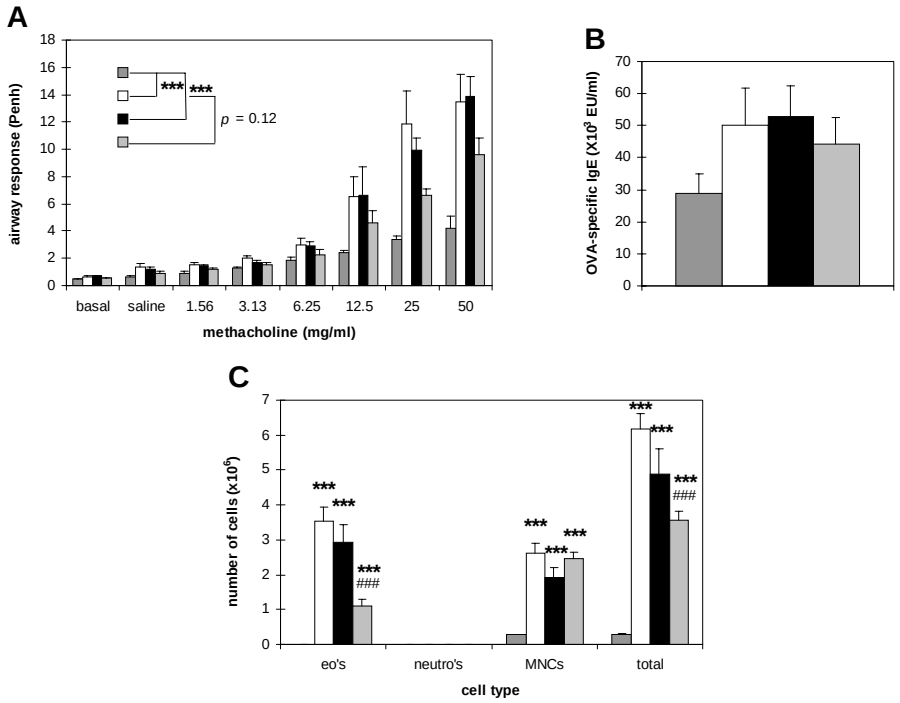

Figure 4: Effects of ICOS blockade in an ongoing murine model of allergic asthma. BALB/c mice were sensitized to OVA adsorbed to alum and challenged with six saline aerosols (*shaded bars*; $n = 8$), six OVA aerosols (*open bars and closed bars*; $n = 8$ per group) or three OVA aerosols followed by three saline aerosols (*lightly shaded bars*; spontaneous resolution group; $n = 8$). Throughout the period of the fourth to sixth aerosol challenge, mice received control treatment (human total IgG, *(lightly) shaded bars and open bars*) or hICOS-Fc (*closed bars)*. Airway responsiveness *in vivo* to aerosolized methacholine (A), OVA-specific IgE levels in serum (B) and the cellular compostion of the BALF (C) were determined at 24 h after the last aerosol. Values are expressed as mean ± SEM. *** $p < 0.001$, as compared with control-treated, saline-challenged animals. ### $p < 0.001$, as compared with control-treated, OVA-challenged animals.

So, blockade of ICOS after the development of asthma-like symptoms in mice that were subjected to ongoing OVA aerosols had no effect on the final airway hyperresponsiveness *in vivo*, OVA-specific IgE levels in serum and inflammatory cell numbers.

DISCUSSION

Asthma patients usually start therapy after the development of airway symptoms, while exposure to environmental allergen mostly continues. Studies on the role of ICOS in murine models of allergic asthma suggest that ICOS-mediated co-stimulation might play a more prominent role in the effector phase than in the initiation phase of Th2-dominated allergic inflammatory responses (26-29). In the present study, we compared the effects of ICOS blockade by hICOS-Fc during the development of asthma-like symptoms by aerosol challenge of OVA-sensitized mice (induction model) or after the development when antigen exposures were continued (ongoing model). In the induction model, treatment of mice with hICOS-Fc resulted in significantly decreased numbers of inflammatory cells in the BALF, but had no effect on non-specific airway hyperresponsiveness and serum OVA-specific IgE levels. In the ongoing model, administration of hICOS-Fc did not alter any of the final asthma-like symptoms. Similarly, IL-5 production by primed lung cells was unaffected upon re-stimulation with OVA in the presence hICOS-Fc e*x vivo*.

With regard to the role of ICOS in the development of asthma-like symptoms, it has been shown that sensitization and aerosol challenge of $ICOS^{-/-}$ mice with OVA resulted in strongly inhibited serum OVA-specific IgE levels compared with wild-type mice (26). However, $ICOS^{-/-}$ mice exhibited similar numbers of eosinophils, lymphocytes and monocytes/macrophages as wild-type mice. In contrast, in a study using a murine model of allergic asthma induced by the *Schistosoma mansoni* parasite, it was demonstrated that administration of ICOS-Ig during sensitization and challenge of C57BL/6 mice, resulted in a reduced number of inflammatory cells in the BALF accompanied by a decrease in the level of IL-5 (27), but no effect on the serum total IgE level was observed. Whereas we used OVA as antigen and BALB/c mice, our results are very much in agreement with this latter study. Tesciuba *et al*. showed that the reduced number of inflammatory cells in the airways was not due to a lack of T-cell priming nor to a defect in Th2 differentiation as determined by unaltered proliferation and Th2 cytokine production of both splenocytes and lung lymphocytes upon antigenic re-stimulation *ex vivo*. Likewise, we observed no difference in IL-5 and IL-10 production between lung cell cultures derived from control- and hICOS-Fc-treated mice re-stimulated with OVA *in vitro* (data not shown). The reduced amount of IL-10 in the BALF of hICOS-Fc-treated mice compared with control-treated mice observed in the present study may be in agreement with the generally

agreed role for ICOS in T-cell IL-10 production (24). However, because IL-10 is a potent anti-inflammatory mediator (32), this is unlikely to explain the reduced amount of BALF inflammatory cells seen in both studies. A unique finding of the present study is that the development of airway hyperresponsiveness, that is dependent on CD4+ T-cells (33, 34), was not affected by the interruption of the ICOS:B7RP-1 pathway. So, T-cell co-stimulation *via* ICOS appears not to be essential for the development of Th2-dominated allergic inflammatory responses in mice.

Interestingly, hICOS-Fc dose-dependently inhibited the production of the prototypical Th2 cytokine IL-5 by TLN cell cultures derived from OVA-sensitized and challenged mice, but lung IL-5 production was unaffected (Figure 2). In a previous study by our laboratory, differences in antigen-induced proliferation and cytokine production between these TLN and lung cell cultures were demonstrated (35). In the supernatant of TLN cell cultures also IL-4 was found upon OVA re-stimulation, whereas lung cells produced only IL-5. Moreover, TLN cells proliferated upon polyclonal stimulation and antigenic re-stimulation, but lung cells failed to do so. Collectively, these data suggest that different populations of antigen-specific T-cells are present in the TLNs and lung tissue in this model that are differentially dependent on ICOS signaling for cytokine production. In agreement herewith is the observation by Garlisi *et al*. that IL-5 mRNA, but not IL-4 mRNA, was detected in BALF T-cells after sensitization and challenge of mice with OVA (36). Likewise, bronchial explant cultures from moderately severe asthma patients produced only IL-5 upon allergen stimulation, whereas peripheral blood mononuclear cells cultures from the same subjects produced also IL-13 and granulocyte-macrophage-colony stimulating factor (37). Moreover, Campbell *et al*. identified specialized “B helper” and “tissue inflammatory” CD4+ T effector subsets that developed concurrently from common naïve precursors during the primary immune response in BALB/c mice (38). These separable populations were distinguishable by their expression of chemoattractant receptors for chemokines expressed in peripheral lymphoid organs and inflamed tissue, respectively. Similarly, two distinct types of human CD4+ and CD8+ memory cells have been identified on the basis of chemokine receptor expression: “central memory” cells present in secondary lymphoid tissues and “effector memory” cells residing in peripheral tissues (*see* 39). In the ongoing murine model, we observed no effects by hICOS-Fc administration on the final asthma-like symptoms. This might indicate that the population of OVA-specific T-cells in the lung that are insensitive to blockade of ICOS *in vitro*, is mainly involved in the maintenance of the ongoing allergic inflammatory Th2 response.

The lack of effect by hICOS-Fc in the ongoing murine model of allergic asthma appears to be in contrast with studies of Coyle and colleagues in murine models of allergic asthma in which effector Th2 cells dominate at the time of ICOS blockade (28, 29). In their first study, CD4+ T-cells of BALB/c mice with a transgenic TCR

that recognizes residues 323-339 of OVA were differentiated *in vitro* towards the Th2 phenotype before adoptive transfer to wild-type BALB/c mice. Administration of ICOS-Ig inhibited the ability of these cells to mediate eosinophilic lung inflammation and airway hyperresponsiveness following airway challenge (28). The generation of Th2 cells *in vitro* may result in effector T-cells with migratory and functional properties that are uncommon *in vivo*. However, in the second study of this group, mice were actively sensitized and aerosol challenged with OVA (29). The authors observed that administration of mAb 12A8 against ICOS solely during the last (seventh) aerosol challenge of OVA-sensitized mice significantly reduced the amount of BALF inflammatory cells and airway hyperresponsiveness, whereas ICOS blockade only at the time of sensitization had little effect upon subsequent airway challenge. The various parameters in this study were measured at 3 h after the last OVA aerosol challenge. When we administered the same dose of the 12A8 mAb in the murine model of ongoing disease, we observed, at 24 h after the last OVA aerosol challenge, no alteration in the final manifestations of allergic asthma, similar to administration of hICOS-Fc (D.T. Deurloo, unpublished observations). Perhaps, ICOS exerts its inhibitory effect in a narrow time window of the T-cell effector phase.

In contrast with this study of Gonzalo *et al*. (29), we sensitized mice to OVA in the presence of alum adjuvant that induces a Th2-dominant response *in vivo* resulting in relatively stronger inflammatory responses compared with immunization without adjuvant (chapters 3 and 5). It is generally acknowledged that not only the mode of sensitization but also the challenge protocol might influence the outcome of an experimental study (23, 40, 41). However, in $ICOS^{-/-}$ mice, it has been shown that immunization with antigen in the presence of alum did not bypass the requirement for ICOS (26, 42, 43). Moreover, the challenge protocols were fairly similar in both studies. Lastly, the genetic background may have had a role in the different results because Gonzalo *et al*. used C57BL/6 mice for their experiments, whereas we made use of BALB/c mice. Future experiments might explain the discrepancies between this study and those of Coyle and colleagues.

Apart from its role in co-stimulation of Th2 responses, a new role for ICOS co-stimulation was recently shown in T-cell tolerance to respiratory allergens (44). Pulmonary dendritic cells (DCs) have been shown to induce the development of allergy-mediating Th2 cells (45) and CD4+ regulatory T-cells -producing primarily IL-10 and low levels of IL-4 (46)- that inhibit Th2 cells, thus protecting against asthma development (44, 47). The specific signals that preferentially induce development of CD4+ regulatory T-cells rather than Th2 cells are not entirely clear, but may involve IL-10 production by DCs (44, 47). However, both the development and inhibitory function of CD4+ regulatory T-cells were shown to be dependent on the ICOS:B7RP-1 signaling pathway (44). In these studies, CD4+ regulatory T-cells

were induced by intranasal exposure to OVA that inhibited the development of asthma manifestations upon subsequent re-challenge with OVA. Adoptive transfer of these CD4+ regulatory T-cells into OVA-sensitized mice potently inhibited asthma-like symptoms following aerosol challenge (44). Although it is unclear if regulatory T-cells play a role in our models in which mice are not previously exposed to intranasal OVA, blockade of ICOS may on the one hand alleviate airway symptoms by the inhibition of Th2 cells and on the other hand aggravate symptoms by the inhibition of regulatory T-cells.

The experimental murine model of ongoing disease has been designed in an attempt to more closely resemble the clinical situation in which patients start treatment after the onset of airway symptoms and demonstrates the difficulty of reversing established symptoms with ongoing antigen exposures in mice. Whereas CTLA4-IgG and the "gold-standard" dexamethasone were partially effective in this model in that serum antigen-specific IgE levels and BALF eosinophilia could be reduced (23), hICOS-Fc had no effect. In addition, the combined administration of CTLA4-IgG and mAb 12A8 against ICOS did not have a synergistic effect (data not shown). Future studies using the ongoing model may elucidate which (combination of) other signals are required for allergic inflammatory responses dominated by antigen-experienced CD4+ T-cells.

ACKNOWLEDGEMENTS

D.T.D. and E.C.A.M.v.E. are supported by Research Grant 3.2.97.09 from The Netherlands Asthma Foundation. We thank Miranda van Berkel (Department Immunology, University Medical Center, Utrecht, The Netherlands) for expert FACS analysis. We also thank Dr. C.P.M. Broeren (Department of Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands) for kindly providing B7RP-1 transfected CHO cells.

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Chapter 5

CTLA4-IgG can induce partial tolerance in murine models of allergic asthma

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ABSTRACT

In the present study, we investigated if blockade of T-cell co-stimulation during the aerosol challenge period of antigen-sensitized mice results in long-term amelioration of asthma-like symptoms that is, not only immediately after challenge but also upon antigen re-challenge after serum clearance of experimental treatment. BALB/c mice were sensitized to alum-adsorbed ovalbumin (OVA) and challenged with saline or OVA aerosols. Before the first aerosol challenge, mice received control treatment, anti-CD40 ligand (CD40L) mAb (MR1), murine CTLA4-IgG or the combination of anti-CD40L mAb and CTLA4-IgG. In control-treated animals, OVA-challenge induced airway hyperresponsiveness (AHR) *in vivo* and up-regulated levels of serum OVA-specific IgE compared with saline-challenged mice. CTLA4-IgG prevented both parameters, whereas anti-CD40L mAb solely prevented the up-regulation of serum OVA-IgE levels. Eleven weeks later, after serum clearance of treatments, OVA re-challenge induced AHR, up-regulated levels of serum OVA-IgE and the influx of eosinophils in the broncho-alveolar lavage fluid (BALF) in control-treated animals compared with saline-challenged mice. Only CTLA4-IgG-treated mice demonstrated tolerance with respect to the number of eosinophils in the BALF induced by OVA re-challenge (46% inhibition). Interestingly, in a "mild" model of allergic asthma, in which mice are sensitized without adjuvant and challenged with lower doses of OVA, CTLA4-IgG had a long-term effect on both OVA-specific IgE levels and BALF esoinophilia (69% and 80% inhibition, respectively). So, CTLA4-IgG can induce partial tolerance in OVA-induced murine models of allergic asthma, its effectiveness being dependent on the "severity" of the model used.

INTRODUCTION

Allergic asthma is a complex inflammatory disease of the lung characterized by reversible airflow obstruction, persistent airway hyperresponsiveness and chronic airway inflammation. As a result of the chronic inflammation, airway tissue is continuously being injured and healed resulting in structural changes (called remodeling) of airway tissue that further aggravates the disease (1).

Allergen-specific T helper (Th) type 2 cells play a pivotal role in the pathogenesis and progression of allergic asthma by orchestrating the inflammatory response (2). Th2 cells are characterized by the array of cytokines they secrete, such as IL-4, IL-5, IL-9, IL-10 and IL-13, which are optimized to combat parasite infections (3). However, Th2 cells are developed undesirably in allergic disorders such as asthma, their cytokines causing airway symptoms either directly or mediated by other immune cells and their mediators (4). Therefore, silencing the allergen-specific T-cells, preferably not temporarily, but long lasting, is an interesting therapeutic approach.

One therapeutic strategy to silence allergen-specific CD4+ T-cells is the prevention of optimal activation by blocking their co-stimulatory requirements. For optimal activation, naïve CD4+ T-cells require nonspecific co-stimulatory signals in addition to the antigen-specific signal conferred by the T-cell receptor (5). CD28 is the primary co-stimulatory molecule, present on the surface of naïve T-cells. Upon interaction with its ligands B7-1 (CD80) or B7-2 (CD86) on antigen-presenting cells (APCs) CD28 tranduces a potent signal that enhances T-cell proliferation and cytokine secretion and sustains the T-cell response. The second receptor for the B7-1 and B7-2 ligands, CTLA4 (CD152), is expressed on activated T-cells and delivers an inhibitory signal to terminate the T-cell response (6). Another well-known pathway of T-cell co-stimulation is CD40 ligand (CD40L; CD154) expressed on activated T-cells that interacts with CD40 found on many cell types, including APCs. In addition to evidence for direct T-cell co-stimulation *via* CD40L, triggering of CD40 contributes indirectly to T-cell co-stimulation by the upregulation of the B7-1 and B7-2 expression on APCs, thus enhancing T-cell activation through CD28-mediated signaling (7).

In experimental murine models of allergic asthma, blocking the CD28/CTLA4:B7-1/B7-2 pathway of T-cell co-stimulation –not only during antigen sensitization (8), but also solely during the challenge period (8-12) and even in an ongoing response (13)- inhibits asthma-like symptoms such as airway hyperresponsiveness *in vivo*, the levels of antigen-specific IgE in serum and the influx of eosinophils in the broncho-alveolar lavage fluid (BALF). The importance of the CD40L:CD40 pathway in the induction of asthma-like symptoms has only been investigated in mice deficient in either CD40L or CD40 (14-16). Because ligation of CD40 on B-cells, together with IL-4 (17), is critical for their activation and the induction of IgE isotype switching (18), the absence of (allergen-specific) IgE in

serum was anticipated in all of these studies. However, conflicting results were obtained regarding the involvement of the CD40L:CD40 pathway in the development of airway hyperresponsiveness and BALF eosinophilia (14-16).

In the above-mentioned studies, the immediate effects of the lack of a T-cell co-stimulatory pathway -either by blockade or gene deficiency- on the induction of manifestations of allergic asthma in mice were investigated. Studies *in vitro*, however, have demonstrated that in the absence of co-stimulation, antigen-stimulated T-cells besides being not optimally activated are also rendered anergic that is, unresponsive to antigenic re-stimulation in the presence of co-stimulation (19). *In vivo*, blockade of T-cell co-stimulation around the time of engraftment was shown to allow for long-term allograft survival in several transplantation models (*see* 20). So, blockade of T-cell co-stimulation not only results in the immediate suppression of the immune response but in some cases induces long-term antigen-specific tolerance. Short-lived treatment of asthma patients that results in tolerance to the inhalant allergen and hence long-lasting improved clinical status would be a very attractive therapeutic approach. However, the long-term effects of T-cell co-stimulation blockade during the development of antigen-induced asthma-like symptoms in mice have not been studied so far.

Therefore, in the present study, we investigated if blockade of T-cell co-stimulation during the aerosol challenge period of antigen-sensitized mice results in long-term amelioration of asthma-like symptoms that is, not only immediately after the aerosol challenge period but also upon antigen re-challenge after serum clearance of experimental treatment. A number of studies *in vitro* and *in vivo* on the induction of antigen-specific tolerance have demonstrated that blockade of both the CD28/CTLA4:B7-1/B7-2 and the CD40L:CD40 pathway is more effective than blockade of either pathway alone (21-25). Therefore, we compared the immediate and long-term effects of a monoclonal antibody (mAb) against CD40L (MR1), murine CTLA4-IgG or the combination of these treatments in an ovalbumin (OVA)-induced murine model of allergic asthma.

After the first series of OVA aerosol challenges, CTLA4-IgG -in the absence or presence of anti-CD40L mAb- prevented both airway hyperresponsiveness *in vivo* and the up-regulation of OVA-specific IgE levels in serum, whereas anti-CD40L mAb alone solely prevented the up-regulation of serum OVA-specific IgE levels. We are the first to demonstrate that, 11 wk later, after serum clearance of treatments, only CTLA4-IgG-treated mice demonstrated tolerance with respect to the number of eosinophils in the BALF induced by OVA re-challenge. Interestingly, in a “mild” model of allergic asthma, in which mice are sensitized without adjuvant and challenged with low doses of OVA, the long-term effect of CTLA4-IgG was not limited to the inhibition of BALF eosinophilia but extended to reduced levels of OVA-specific IgE in serum. So, CTLA4-IgG can induce tolerance to some of the

OVA-induced asthma-like symptoms in mice, its effectiveness being dependent on the "severity" of murine model used.

MATERIALS AND METHODS

Animals

Specific pathogen-free male BALB/c mice were obtained from the Central Laboratory Animal Institute (Utrecht, The Netherlands). The animals were used at 6-9 wk of age and housed in macrolon cages in a laminar flow cabinet and provided with food and water *ad libitum*. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments as described in DEC-fsb protocol 98.23.

Antigen sensitization and challenge

For the experiment in the more "severe" model of allergic asthma, all mice were sensitized to OVA (chicken egg albumin, crude grade V, Sigma, St. Louis, MO, USA). Systemic sensitization was performed by two intraperitoneal (i.p.) injections of 10 μg OVA adsorbed onto 2.25 mg aluminum hydroxide (alum; ImjectAlum, Pierce, Rockford, IL, USA) in 0.1 ml pyrogen-free saline on day 0 and 7. On day 28, 31 and 34 mice were exposed to a saline aerosol or an OVA aerosol (10 mg/ml in pyrogen-free saline) challenge in a plexiglass exposure chamber (5 liter) for 20 min. On day 112, 115 and 118 (11 wk later), saline-exposed mice received either three saline aerosols or three OVA aerosol challenges ("secondary responses group") and OVA-challenged mice were re-challenged with three OVA aerosols (*see* Table 1).

For the experiment in the "mild" model of allergic asthma, all mice were systemically sensitized to OVA without adjuvant by seven i.p. injections of 10 μg OVA in 0.5 ml pyrogen-free saline on day 0, 2, 4, 7, 9, 11 and 14 (one injection per day). On day 42-49, mice were exposed to a saline aerosol or an OVA aerosol (2 mg/ml in pyrogen-free saline) challenge in a plexiglass exposure chamber (5 liter) for 5 min on consecutive days (one aerosol per day). On day 127-134 (11 wk later), saline-exposed mice received eight saline aerosols and OVA-challenged mice were re-challenged with eight OVA aerosols (*see* Table 2).

The aerosols were generated by nebulizing an OVA solution or saline only using a Pari LC Star nebulizer (PARI Respiratory Equipment, Richmond, VA, USA; particle size 2.5–3.1 μm) driven by compressed air at a flow rate of 6 liters/min.

Experimental treatment protocols

In each experiment, mice received a single intravenous administration of experimental treatment shortly before the first aerosol. As indicated in Tables I and II, one of the four following treatments, dissolved in 0.2 ml pyrogen-free saline, was administered:

a) control treatment (350 μg Armenian hamster IgG control antibody [Ha4/8] and 280 μg human total IgG), b) 350 μg hamster anti-mouse CD40L mAb (MR1) and 280 μg total human IgG, c) 280 μg murine CTLA4-IgG1 and 350 μg Ha4/8 or d) 350 μg MR1 and 280 μg murine CTLA4-IgG1. The half-life of the MR1 mAb in BALB/c mice is about 8.4 d (26). Sera of mice that received either murine CTLA4-IgG or human total IgG, contained less than 0.3 μg/ml of the respective treatment at the time of sacrifice as determined by ELISA. All animals were sacrificed at 24 h after the last aerosol re-challenge (on day 119 [more "severe" model], day 135 ["mild" model]).

The MR1 and Ha4/8 hamster antibodies were kindly provided by Biogen (Cambridge, MA, USA). Murine CTLA4-IgG fusion protein composed of murine CTLA4 fused *via* an ECD-immunoglobulin junction to the hinge and Fc regions of human IgG1 as described in detail previously (11) was kindly provided by Genentech (South San Francisco, CA, USA). Human total IgG was purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA) and dissolved in pyrogen-free saline. This solution was treated with 10% (v/v) polymyxin B-agarose (Sigma; washed twice with saline) for 1 h at 4°C to remove lipopolysaccharide. After incubation, agarose beads were removed by centrifugation, and the supernatant was sterilized. The endotoxin level in all treatment preparations was less than 1 endotoxin U/mg.

Measurement of airway responsiveness in vivo

Airway responsiveness was measured in conscious, unrestrained mice at 24 h after the last aerosol challenge (on day 35 [more "severe" model] or day 50 ["mild" model]) and at 24 h after the last re-challenge (on day 119 [more "severe" model], day 135 ["mild" model]) by recording respiratory pressure curves in response to inhaled nebulized methacholine (acetyl-β-methylcholine chloride; Sigma) using barometric whole-body plethysmography (Buxco; EMKA Technologies, Paris, France). Airway responses were expressed in enhanced pause (Penh), an index of airway obstruction as described in detail previously (27). Briefly, mice were placed in a whole-body chamber and pressure differences between this chamber and a reference chamber were recorded. After baseline Penh values were obtained for 3 min and averaged, animals were exposed to a saline aerosol and a series of methacholine aerosols (solutions doubling in concentration, ranging from 1.56–50 mg/ml in saline). Aerosols were generated by a Pari LC Star nebulizer for 3 min and after each nebulization readings were taken for 3 min and averaged.

Determination of OVA-specific IgE levels in serum

In the experiment using the more "severe" model of allergic asthma, approximately 0.25 ml blood was recovered from mice by an incision the tail vein at day 39 (4 d after the last aerosol challenge). In both experiments, after measurement of airway

responsiveness *in vivo* at 24 h after the last aerosol re-challenge, mice were sacrificed by i.p. injection of 1 ml 10% urethane (Sigma) in sterile saline and bled by cardiac puncture. Serum was prepared from blood samples and stored at -20°C until determination of OVA-specific IgE levels by ELISA. Briefly, 96-wells maxisorp microtiter plates (Nunc A/S, Roskilde, Denmark) were coated overnight at 4°C with 1 μg/ml rat anti-mouse IgE mAb (clone R35-72, PharMingen, San Diego, CA, USA) diluted in phosphate-buffered saline (PBS). The next day, the ELISA was performed at room temperature using ELISA buffer (PBS containing 0.5% bovine serum albumin [Sigma], 2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.05% Tween-20 [Merck, Whitehouse Station, NJ, USA], pH 7.2) for blocking and dilution of samples, standard and reagents and PBS containing 0.05% Tween-20 for washing between incubations. After blocking of wells for 1 h, serum samples and a duplicate dilution series of an OVA-specific reference serum (starting 1:40) were added to the wells and incubated for 2 h. An OVA-specific IgE reference serum was obtained by sensitization and challenge of mice with OVA as above-described for the more "severe" model of allergic asthma and arbitrarily assigned a value of 1,000 experimental units/ml (EU/ml). Hereafter, 1 μg/ml of OVA labeled to digoxigenin (DIG) by a DIG protein labeling kit (Roche Diagnostics, Basel, Switzerland) was added for 1.5 h, followed by incubation with 1:500 diluted anti-DIG-Fab fragments coupled to horseradish peroxidase (Roche Diagnostics) for 1 h. Color development was performed with *o*-phenylenediamine-dichloride substrate (0.4 mg/ml, Sigma) and 4 mM H_2O_2 in PBS and the reaction was stopped by adding 4 M H_2SO_4. The optical density was read at 490 nm, using a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA). Results were analyzed with Microplate Manager PC software (Bio-Rad Laboratories). The lower detection limit of the ELISA was 4 EU/ml IgE.

Analysis of the BALF

BAL was performed immediately after bleeding of the mice as described in detail previously (28). Briefly, the airways were lavaged five times through a tracheal cannula with 1 ml saline at 37°C. The recovered fluid of the five lavages was pooled and cells were pelleted (387 × *g*, 4°C, 10 min), and resuspended in 0.15 ml cold PBS. The total number of cells in the BALF was counted using a Bürker-Türk counting-chamber (Karl Hecht Assistent KG, Sondheim/Röhm, Germany). For differential BALF cell counts, cytospin preparations were made (15 × *g*, 4°C, 5 min) using a cell container (Kendro Heraues Instruments, Asheville, NC, USA), and cells were fixed and stained with Diff-Quick (Dade AG, Düdingen, Switzerland). Per cytospin, at least 400 cells were counted and differentiated into mononuclear cells (monocytes, macrophages and lymphocytes), eosinophils and neutrophils by standard morphology and staining characteristics. The investigator counting the cells was blinded to the treatment groups.

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). The airway dose-response curves to methacholine were statistically analyzed by a general linear model of repeated measurements followed by *post-hoc* comparison between groups. Data were log10 transformed before analysis to equalize variances in all groups. Statistical analysis on BALF cell counts was performed using the non-parametric Mann Whitney *U* test. For all other parameters, results were statistically analyzed using a Student's *t* test (two-tailed, homosedastic). Differences between groups were considered statistically significant at the $p < 0.05$ level. Statistical analyses were performed using SPSS for Windows version 10.0.5 (SPSS, Chicago, IL, USA).

RESULTS

Table 1 shows the design of the experiment to determine the long-term effects of blocking T-cell co-stimulation during the development of antigen-induced asthma-like symptoms in mice. Upon sensitization to the experimental allergen OVA, OVA-specific memory T-cells developed (primary response). Subsequently, mice received experimental treatment and were challenged with an OVA aerosol to develop asthma-like symptoms (secondary responses). Finally, after serum clearance of the treatments, mice were re-challenged with OVA to re-induce airway symptoms (tertiary responses). One might speculate that in OVA-sensitized, control-treated mice, tertiary responses after OVA re-challenge are higher compared with the secondary responses induced by the first series of OVA aerosol challenges (Group 2, *see* Table 1). Therefore, to be able to compare the magnitude of the tertiary responses with time-matched secondary allergic responses, Group 6 was included in the experiment consisting of mice that received saline aerosols directly after administration of control treatment and OVA aerosols during the re-challenge period (hereafter referred to as the "secondary responses group").

Table 1: Experimental design to determine the long-term effects of blocking T-cell co-stimulation during the development of antigen-induced asthma-like symptoms in mice.

Group	Sensitization (Prim response)	Treatment before aerosol 1	Challenge aerosol 1-3 (Sec responses)	Re-challenge, 11 wk later aerosol 4-6 (Tertiary responses)
1	OVA/alum	Ham IgG and h total IgG	Saline	Saline
2	OVA/alum	Ham IgG and h total IgG	OVA	OVA
3	OVA/alum	Anti-CD40L and h total IgG	OVA	OVA
4	OVA/alum	CTLA4-IgG and hamIgG	OVA	OVA
5	OVA/alum	CTLA4-IgG and anti-CD40L	OVA	OVA
6	OVA/alum	Ham IgG and h total IgG	Saline	OVA (Sec responses group)

Definition of abbreviations: h, human; ham, hamster. Mice were sensitized, challenged and treated as described in MATERIALS AND METHODS. Each experimental group consisted of eight animals.

Immediate and long-term effects of T-cell co-stimulatory blockade on airway hyperresponsiveness in vivo

First, the immediate effects of T-cell co-stimulatory blockade on OVA-induced airway hyperresponsiveness *in vivo* were determined at 24 h after the last aerosol challenge. The airway responses to doubling concentrations of aerosolized methacholine were measured in conscious, unrestrained mice by whole-body plethysmography. In control-treated mice, OVA aerosol challenge of OVA-sensitized mice resulted in hyperresponsiveness to methacholine because the complete dose-response curve (DRC) to methacholine of this group of mice was significantly different ($p < 0.01$) from that of saline-challenged mice. For brevity, only the maximal responses are shown in Figure 1A. Administration of the anti-CD40L mAb MR1 before the first OVA aerosol challenge had no effect on the development of airway hyperresponsiveness. In contrast, murine CTLA4-IgG, in the absence or presence of anti-CD40L mAb, prevented the development of OVA-induced airway hyperresponsiveness ($p < 0.05$ and $p < 0.05$, respectively). At this time point of the experiment, the secondary responses group (Group 6, *see* Table 1) was a duplicate of Group 1. Therefore, the airway responses of mice from these two groups are averaged and depicted together as one bar in Figure 1A.

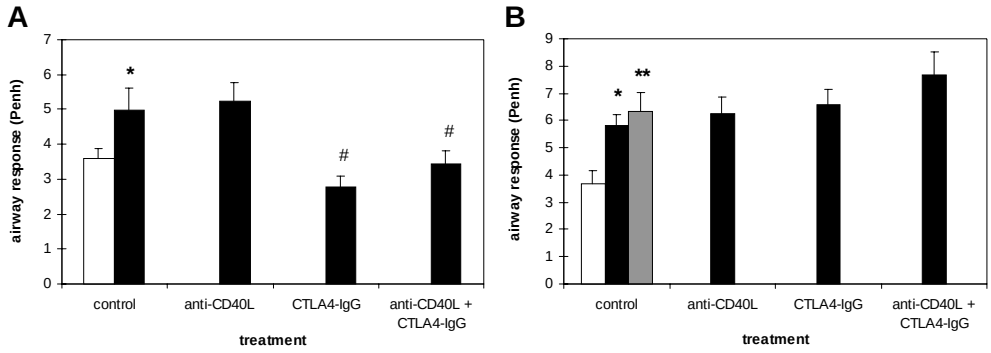

Figure 1: Immediate and long-term effects of T-cell co-stimulatory blockade on airway hyperresponsiveness *in vivo*. Airway responsiveness *in vivo* to aerosolized methacholine was measured in conscious, unrestrained mice by whole-body plethysmography at 24 h after the last aerosol challenge (A) and at 24 h after the last re-challenge (B). For brevity, only the maximal responses (to the highest concentration of the methacholine DRC (50 mg/ml)) are shown. (A) BALB/c mice were sensitized to OVA adsorbed to alum and challenged with three saline aerosols (*open bar*; $n = 16$) or three OVA aerosols (*closed bars*; $n = 8$-9 per group). Shortly before the first aerosol challenge, mice received control treatment, anti-CD40L mAb, mCTLA4-IgG or the combination of anti-CD40L mAb and CTLA4-IgG. (B) Eleven weeks later, after serum clearance of treatments, saline-challenged mice were re-challenged with three saline aerosols (*open bar*; $n = 7$) or three OVA aerosols (*shaded bar*; $n = 8$) and OVA-challenged mice were re-challenged with three OVA aerosols (*closed bars*; $n = 7$-9 per group). Values are expressed as mean ± SEM. * $p < 0.01$ and ** $p < 0.005$, as compared with saline-challenged animals of the same treatment group. # $p < 0.05$, as compared with control-treated, OVA-challenged animals.

Eleven weeks after the last aerosol challenge, when the treatments had been cleared from the sera of mice, animals were re-challenged with either three saline or three OVA aerosols (*see* Table 1) to determine the long-term effects of blockade of T-cell co-stimulation on asthma-like symptoms re-induced by OVA re-challenge. Airway hyperresponsiveness to methacholine *in vivo* was measured again at 24 h after the last aerosol re-challenge. In control-treated mice, OVA aerosol re-challenge of OVA-sensitized and challenged mice resulted in a significantly different maximal airway response compared with saline re-challenged mice ($p < 0.01$) (Figure 1B). The secondary responses group showed a similar increase in the maximal airway response compared with saline re-challenged animals ($p < 0.05$). Earlier administration of anti-CD40L mAb, CTLA4-IgG or the combination of these treatments before the development of asthma manifestations by the first series of OVA aerosols had no long-term effect on airway hyperresponsiveness induced by OVA re-challenge.

So, administration of anti-CD40L mAb had no immediate effect on OVA-induced airway hyperresponsiveness *in vivo* to methacholine in mice. In contrast, CTLA4-IgG - in the absence or presence of anti-CD40L mAb - prevented the development of airway hyperresponsiveness, but had no long-term effect on this parameter. The magnitude of airway hyperresponsiveness of mice from the secondary responses group was very similar to the level of tertiary response hyperresponsiveness.

Immediate and long-term effects of T-cell co-stimulatory blockade on the up-regulation of OVA-specific IgE levels in serum

Four days after the last aerosol challenge, blood was withdrawn from mice to determine the immediate effects of T-cell co-stimulatory blockade on the OVA-induced up-regulation of antigen-specific IgE in serum. In control-treated mice, OVA aerosol challenge of OVA-sensitized mice resulted in a significant up-regulation of OVA-specific IgE compared with saline-challenged animals (168% increase, $p < 0.0005$) (Figure 2A). Administration of anti-CD40L mAb, CTLA4-IgG or the combination of these treatments before the first OVA aerosol challenge inhibited the up-regulation of serum OVA-specific IgE significantly (70% inhibition, $p < 0.005$, 55% inhibition, $p < 0.05$ and 88% inhibition, $p < 0.0005$, respectively). Because at this time point of the experiment the secondary responses group was a duplicate of Group 1, the levels of OVA-specific IgE in the sera of mice from these two groups are averaged and depicted as one bar in Figure 2A.

After measurement of airway responsiveness *in vivo* at 24 h after the last aerosol re-challenge, mice were killed and bled by cardiac puncture to determine the long-term effects of T-cell co-stimulatory blockade on OVA-specific IgE levels in serum induced by OVA re-challenge. In control-treated mice, OVA re-challenge of OVA-sensitized and challenged mice resulted in a significant up-regulation of OVA-specific

IgE levels compared with saline re-challenged animals (334% increase, $p < 0.05$) (Figure 2B). The secondary responses group showed a similar up-regulation of serum OVA-specific IgE levels compared with saline re-challenged animals (415% increase, $p < 0.01$). Earlier administration of anti-CD40L mAb, CTLA4-IgG or the combination of these treatments before the development of asthma manifestations by the first series of OVA aerosols had no significant long-term effect on the levels of OVA-specific IgE in serum induced by OVA re-challenge.

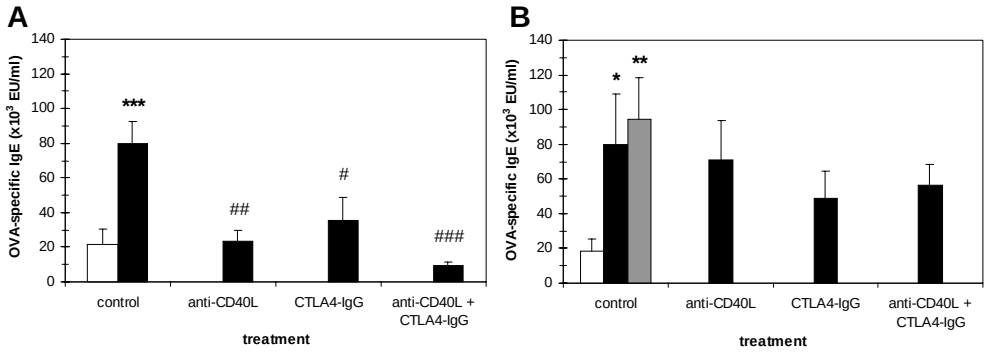

Figure 2: Immediate and long-term effects of T-cell co-stimulatory blockade on the up-regulation of OVA-specific IgE levels in serum. OVA-specific IgE was determined in the sera of mice at 24 h after the last aerosol challenge (A) and at 24 h after the last re-challenge (B). (A) BALB/c mice were sensitized to OVA adsorbed to alum and challenged with three saline aerosols (*open bar*; $n = 12$) or three OVA aerosols (*closed bars*; $n = 5$-6 per group). Shortly before the first aerosol challenge, mice received control treatment, anti-CD40L mAb, mCTLA4-IgG or the combination of anti-CD40L mAb and CTLA4-IgG. (B) Eleven weeks later, after serum clearance of treatments, saline-challenged mice were re-challenged with three saline aerosols (*open bar*; $n = 8$) or three OVA aerosols (*shaded bar*; $n = 8$) and OVA-challenged mice were re-challenged with three OVA aerosols (*closed bars*; $n = 7$-9 per group). Values are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0005$, as compared with saline-challenged animals of the same treatment group. # $p < 0.05$, ## $p < 0.005$ and ### $p < 0.0005$, as compared with control-treated, OVA-challenged animals.

So, administration of anti-CD40L mAb, CTLA4-IgG or the combination of these treatments inhibited the OVA-induced up-regulation of serum antigen-specific IgE levels in mice. However, none of these treatments had a long-term effect on this parameter. The secondary responses group showed a similar up-regulation of serum OVA-specific IgE levels as the animals with tertiary responses.

Long-term effects of T-cell co-stimulatory blockade on the number of eosinophils in the BALF

Lavage of the lungs, to determine the number of various cell types that infiltrated the airway lumen, can only be performed in euthanasized mice. Therefore, only the long-term effects of T-cell co-stimulatory blockade on the cellular composition of the BALF could be determined in this experiment. Because there are no or sometimes

only a few eosinophils in OVA-sensitized, saline-challenged mice, the open bar representing the group of control-treated, saline re-challenged animals cannot be seen in Figure 3. However, OVA re-challenge of OVA-sensitized and challenged mice resulted in a significant increase in the number of eosinophils in the BALF compared with saline re-challenged animals ($p < 0.0001$). The secondary responses group showed a similar increase in the number of BALF eosinophils compared with saline re-challenged animals ($p < 0.0005$). Earlier administration of anti-CD40L mAb before the development of asthma manifestations by the first series of OVA aerosols had no long-term effect on the number of eosinophils in the BALF induced by OVA re-challenge. In contrast, CTLA4-IgG-treated mice showed significantly reduced numbers of BALF eosinophils upon OVA re-challenge compared with control-treated mice (46% inhibition, $p < 0.01$). In the presence of anti-CD40L mAb, CTLA4-IgG attenuated the number of eosinophils induced by OVA re-challenge compared with control-treated mice, but this reduction did not reach statistical significance (35% inhibition, $p = 0.14$).

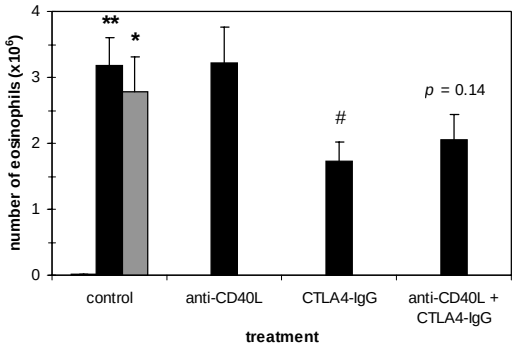

Figure 3: Long-term effects of T-cell co-stimulatory blockade on the number of eosinophils in the BALF. The number of eosinophils in the BALF was determined at 24 h after the last aerosol re-challenge. BALB/c mice were sensitized to OVA adsorbed to alum and challenged with three saline aerosols or three OVA aerosols. Shortly before the first aerosol challenge, mice received control treatment, anti-CD40L mAb, mCTLA4-IgG or the combination of anti-CD40L mAb and CTLA4-IgG. Eleven weeks later, after serum clearance of treatments, saline-challenged mice were re-challenged with three saline aerosols (*open bar*; $n = 8$) or three OVA aerosols (*shaded bar*; $n = 8$) and OVA-challenged mice were re-challenged with three OVA aerosols (*closed bars*; $n = 7$-9 per group). Values are expressed as mean ± SEM. * $p < 0.0005$ and ** $p < 0.0001$, as compared with saline-challenged animals of the same treatment group. # $p < 0.01$, as compared with control-treated, OVA-challenged animals.

So, earlier administration of anti-CD40L mAb before the development of asthma-like symptoms in mice had no long-term effect on airway eosinophilia. In contrast, earlier administration of CTLA4-IgG inhibited the number of BALF eosinophils induced by OVA re-challenge. The number of eosinophils in the BALF of mice

from the secondary responses group was very similar to the number observed in animals with tertiary response airway eosinophilia.

Long-term effects of T-cell co-stimulatory blockade in a "mild" model of allergic asthma

For the above-described experiment, animals were sensitized to OVA in the presence of alum adjuvant that induces a Th2-dominant response *in vivo* and challenged with high doses of OVA. This protocol results in relatively strong inflammatory responses that appear to represent a more "severe" type of allergic asthma. In our laboratory, we also use a "mild" model of allergic asthma in which mice are sensitized to OVA without adjuvant and challenged with lower doses of antigen. After the last OVA aerosol challenge, mice subjected to this protocol, show a similar extent of airway hyperresponsiveness *in vivo*, but animals display only ~ one tenth of serum OVA-specific IgE levels and less than half of the number of BALF eosinophils observed in the more "severe" murine model of allergic asthma. Interestingly, previous studies by others and us have demonstrated that the various models respond differently to modulation in both a quantitative and qualitative way (13, 29, 30). Therefore, we determined the long-term effects of T-cell co-stimulatory blockade in a "mild" murine model of allergic asthma as well. Table 2 shows the design of this experiment.

Table 2: Experimental design to determine the long-term effects of blocking T-cell co-stimulation during the development of antigen-induced asthma-like symptoms in mice in a "mild" model of allergic asthma.

Group	Sensitization (Prim response)	Treatment before aerosol 1	Challenge aerosol 1-8 (Sec responses)	Re-challenge, 11 wk later aerosol 9-16 (Tertiary responses)
1	OVA w/o adj	Ham IgG and h total IgG	Saline	Saline
2	OVA w/o adj	Ham IgG and h total IgG	OVA	OVA
3	OVA w/o adj	Anti-CD40L and h total IgG	OVA	OVA
4	OVA w/o adj	CTLA4-IgG and ham IgG	OVA	OVA
5	OVA w/o adj	CTLA4-IgG and anti-CD40L	Saline	Saline
6	OVA w/o adj	CTLA4-IgG and anti-CD40L	OVA	OVA

Definition of abbreviations: adj, adjuvant; h, human; ham, hamster. Mice were sensitized, challenged and treated as described in MATERIALS AND METHODS. Each experimental group consisted of eight animals.

Airway hyperresponsiveness in vivo

In accordance with the more "severe" model of allergic asthma, earlier administration of anti-CD40L mAb, CTLA4-IgG or the combination of these treatments before the development of asthma manifestations by the first series of OVA aerosols had also in this "mild" model of allergic asthma no long-term effect on airway hyperresponsiveness induced by OVA re-challenge (data not shown).

OVA-specific IgE levels in serum

After measurement of airway responsiveness *in vivo* at 24 h after the last aerosol re-challenge, mice were killed and bled by cardiac puncture to determine the long-term effects of T-cell co-stimulatory blockade on OVA-specific IgE levels in serum. In control-treated mice, OVA re-challenge of OVA-sensitized and challenged mice resulted in a significant up-regulation of OVA-specific IgE levels compared with saline re-challenged animals (513% increase, $p < 0.01$) (Figure 4A). Earlier administration of anti-CD40L mAb before the development of asthma manifestations by the first series of OVA aerosols had no long-term effect on the levels of OVA-specific IgE in serum induced by OVA re-challenge. In contrast, CTLA4-IgG, in the absence or presence of anti-CD40L mAb, significantly reduced the up-regulation of OVA-specific IgE upon OVA re-challenge compared with control-treated mice (69% inhibition, $p < 0.05$ and 61% inhibition, $p < 0.05$, respectively). Treatment of mice with the combination of anti-CD40L mAb and CTLA4-IgG had no long-term effect by itself (Group 5, *see* Table 2) on the levels of OVA-specific IgE as similar levels were found in the sera of control-treated, saline re-challenged mice.

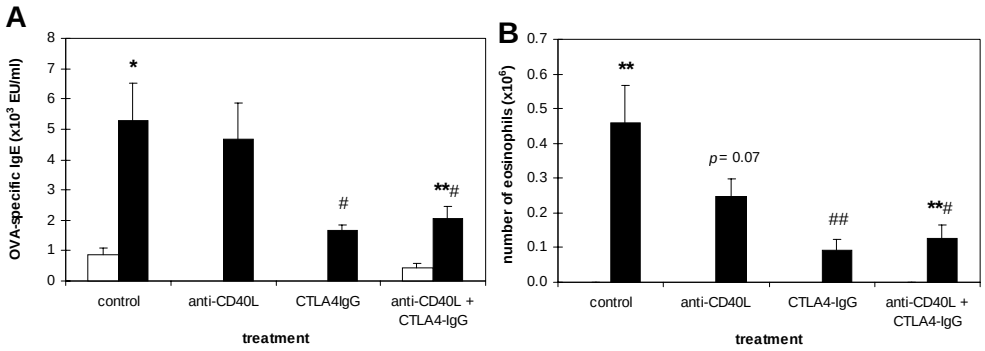

Figure 4: Long-term effects of T-cell co-stimulatory blockade in a "mild" murine model of allergic asthma. OVA-specific IgE in the sera of mice (A) and the number of eosinophils in the BALF (B) were determined at 24 h after the last aerosol re-challenge. BALB/c mice were sensitized to OVA without adjuvant and challenged with eight saline aerosols or eight OVA aerosols. Shortly before the first aerosol challenge, mice received control treatment, anti-CD40L mAb, mCTLA4-IgG or the combination of anti-CD40L mAb and CTLA4-IgG. Eleven weeks later, after serum clearance of treatments, saline-challenged mice were re-challenged with eight saline aerosols (*open bars*; $n = 6$ per group) and OVA-challenged mice were re-challenged with eight OVA aerosols (*closed bars*; $n = 5$-6 per group). Values are expressed as mean ± SEM. * $p < 0.01$ and ** $p < 0.005$, as compared with saline-challenged animals of the same treatment group. # $p < 0.05$ and ## $p < 0.01$, as compared with control-treated, OVA-challenged animals.

BALF eosinophilia

Again, no eosinophils were observed in OVA-sensitized, saline re-challenged mice (Figure 4B). However, OVA re-challenge of OVA-sensitized and challenged mice resulted in a significant increase in the number of eosinophils in the BALF compared

with saline re-challenged animals ($p < 0.005$). Earlier administration of anti-CD40L mAb before the development of asthma manifestations by the first series of OVA aerosols attenuated the number of eosinophils in the BALF induced by OVA re-challenge, but this reduction did not reach statistical significance (46% inhibition, $p = 0.065$). In contrast, mice treated with CTLA4-IgG, in the absence or presence of anti-CD40L mAb showed significantly reduced numbers of BALF eosinophils upon OVA re-challenge compared with control-treated mice (80% inhibition, $p < 0.01$ and 73% inhibition, $p < 0.05$, respectively). Treatment of mice with the combination of anti-CD40L mAb and CTLA4-IgG had no long-term effect by itself on the number of eosinophils in the BALF as similar numbers were found in the sera of control-treated, saline re-challenged mice.

So, in a "mild" murine model of allergic asthma, earlier administration of anti-CD40L mAb before the development of asthma-like symptoms had no significant long-term effect on any of the asthma-like symptoms. In contrast, treatment of mice with CTLA4-IgG – in the absence or presence of anti-CD40L mAb- inhibited both the up-regulation of antigen-specific IgE in serum and the number of BALF eosinophils induced by OVA aerosol re-challenge, but had no long-term effect on airway hyperresponsiveness.

DISCUSSION

Blockade of T-cell co-stimulation has been shown not only to result in the immediate suppression of the immune response but to induce in some disease models long-term antigen-specific tolerance as well. Short-lived treatment of asthma patients that results in long-term tolerance to the inhalant allergen and hence long-lasting improved clinical status would be a very attractive therapeutic approach. Therefore, in the present study, we investigated if blockade of T-cell co-stimulation the during the aerosol challenge period of OVA-sensitized mice results in long-term amelioration of asthma-like symptoms that is, not only immediately after the aerosol challenge period but also upon antigen re-challenge after serum clearance of experimental treatment. After the first series of OVA aerosol challenges, CTLA4-IgG prevented both the development of non-specific airway hyperresponsiveness *in vivo* and the up-regulation of OVA-specific IgE levels in serum, whereas anti-CD40L mAb (MR1) solely prevented the up-regulation of serum OVA-specific IgE levels. After serum clearance of treatments, only CTLA4-IgG-treated mice demonstrated tolerance with respect to the number of eosinophils in the BALF induced by OVA re-challenge. Interestingly, in a "mild" model of allergic asthma, in which mice are sensitized without adjuvant and challenged with lower doses of OVA, the long-term effect of CTLA4-IgG was not limited to the inhibition of BALF eosinophilia but extended to reduced levels of OVA-specific IgE in serum.

Previous studies have already shown that blocking the CD28/CTLA4:B7-1/B7-2 pathway in mice - during antigen sensitization (8), solely during the challenge period (8-12) and even in an ongoing response (13)- inhibited the development of airway hyperresponsiveness *in vivo* and the up-regulation of serum IgE levels. In addition, a reduction in airway eosinophilia was observed in all of these studies. Here, we present the first demonstration that in OVA-induced murine models of allergic asthma, CTLA4-IgG induced tolerance to this experimental allergen. Interestingly, tolerance could be induced for airway eosinophilia and OVA-specific IgE but not for non-specific airway hyperresponsiveness. So, whereas CTLA4-IgG inhibits the development of the three afore-mentioned manifestations of allergic asthma, the tolerization of these parameters was dissociated in this study. Therefore, our results suggest that the various CD4+ T-cell-dependent asthma-like symptoms in mice are differentially modulated by CTLA4-IgG administration.

How can the selective tolerization of CD4+ T-cell effector functions by CTLA4-IgG be explained? One possibility is that CTLA4-IgG selectively tolerizes B7-1/B7-2-dependent T-cell signal transduction pathways. For example, in mice deficient for signal transducer and activator of transcription (STAT)-6 (STAT-$6^{-/-}$), airway eosinophilia was found to be partially inhibited in contrast to the complete absence of airway hyperresponsiveness and antigen-specific IgE in these mice (31, 32). These studies suggest that the various T-cell effector functions observed in mice are differentially dependent on STAT-6. Because CD28 has been shown to activate STAT-6 (33), a differential sensitivity to tolerance induction by blockade of the CD28/CTLA4:B7-1/B7-2 pathway of the asthma-like symptoms in mice is possible. Interestingly, CTLA4-Ig induced long-term engraftment of cardiac allografts and donor-specific tolerance in both BALB/c wild-type and STAT-$6^{-/-}$ recipients (34). However, in another study, STAT-6 signaling was shown to be involved, at least in part, in CTLA4-Ig-mediated long-term maintenance of tolerance (35). Moreover, other research groups found that airway hyperresponsiveness and eosinophilia are equally dependent on STAT-6 (36, 37).

Another possibility is that the various manifestations of allergic asthma are controlled by different subsets of (allergen-specific) CD4+ T-cells that might differ in their sensitivity to tolerance induction by CTLA4-IgG. Notably, Campbell *et al*. identified specialized “B helper” and “tissue inflammatory” CD4+ T-cell subsets that developed concurrently from common naive precursors during the primary immune response in BALB/c mice (38). This observation seems to contrast with studies demonstrating that Th1 and Th2 cells polarized *in vitro* can mediate immune responses in peripheral tissues as well as provide B cell help in lymphoid follicles upon adoptive transfer (39, 40). However, analysis of Th1 and Th2 populations at the single cell or clone level has demonstrated remarkable diversity in their cytokine profiles (41). In addition, individual Th1 and Th2 clones have been shown to be

functionally heterogeneous (42). Future studies may clarify whether specialized T-cell subsets are contained within polarized populations of Th1 and Th2 cells. Moreover, the generation of Th2 cells *in vitro* may result in effector T-cells with migratory and functional properties that are uncommon *in vivo*.

In vitro, it was found that the successful induction of unresponsiveness toward soluble antigens with CTLA4-Ig required complete blockade of antigen-induced proliferation during the induction culture (43). Along this line, although CTLA4-IgG greatly reduced the asthma-like symptoms in both the more "severe" and "mild" model of allergic asthma (this study and Ref 11), the symptoms were not fully blocked suggesting that not all OVA-specific CD4+ T-cells were inhibited and hence (selectively) tolerized. These non-tolerized antigen-specific T-cells are likely to be responsible for the residual airway eosinophilia (and OVA-IgE levels) observed upon OVA re-challenge. In addition, the more pronounced long-term effect of CTLA4-IgG on airway eosinophilia in the "mild" model of allergic asthma might be explained by our observation that the development of this parameter is more readily inhibited by CTLA4-IgG in this model compared with the more "severe" model of allergic asthma. This in turn might be clarified by a study demonstrating that the stringent requirement for CD28 in CD4+ T-cell activation can be overcome by higher concentrations of antigen or longer durations of exposure to antigen (44). Interestingly, human CTLA4-Ig inhibited IL-5 and IL-13 production by allergen-exposed bronchial explant cultures derived from mild asthmatics (45), but not from moderately severe asthmatics (46).

However, in both the "mild" and more "severe" murine model of allergic asthma, administration of CTLA4-IgG failed to fully block the development of asthma-like symptoms induced by OVA challenge (this study and Ref 11). Possibly, CTLA4-Ig leaves *in vivo* some B7-1 and B7-2 molecules uncovered (47) that may bind CD28 (and CTLA4). In addition, other molecules than CD28, may provide important co-stimulatory signals to the T-cells in our models. With regard to the CD40L:CD40 pathway of T-cell co-stimulation, studies *in vitro* and *in vivo* have demonstrated that disruption of this pathway inhibits the T-cell response (21-25). Interestingly, an additional or synergistic effect on the induction of antigen-specific tolerance was observed when both the CD28/CTLA4:B7-1/B7-2 pathway and the CD40L:CD40 pathway were blocked in comparison with blockade of either pathway alone in these studies. Therefore, we compared the immediate and long-term effects of blockade of T-cell co-stimulation by anti-CD40L mAb, CTLA4-IgG or the combination of these treatments during the aerosol challenge period of antigen-sensitized mice. The anti-CD40L mAb MR1 inhibited the up-regulation of serum OVA-IgE but had no effect on the development of OVA-induced airway hyperresponsiveness *in vivo*. Because ligation of CD40 on B-cells, together with IL-4 (17), is critical for their activation and the induction of isotype switching to IgE (18), the inhibition of allergen-

specific IgE in serum observed in mice treated with anti-CD40L mAb clearly demonstrates the success of this treatment. The experimental set-ups used in this study did not allow the determination of the immediate effect of this antibody on the development of BALF eosinophilia. However, in separate experiments –using either the "mild" or the more "severe" murine model of allergic asthma-, we observed no effect on BALF eosinophilia induced by OVA-challenge by the same anti-CD40L mAb (D.T. Deurloo and H. Scheerens *et al*., unpublished observations). Moreover, after serum clearance of treatments, anti-CD40L mAb did not add to the tolerance induced by CTLA4-IgG nor had a significant long-term effect by itself. Perhaps the blockade of yet other T-cell co-stimulatory molecule(s) -*e.g.* inducible T-cell co-stimulator (ICOS)- in combination with CTLA4-Ig is required to completely block T-cell activation in mice during the aerosol challenge period and to induce antigen-specific tolerance to all manifestations of allergic asthma.

The importance of the CD40L:CD40 pathway in the induction of asthma-like symptoms has only been investigated in mice deficient in either CD40L or CD40 (14-16). Comparing CD40-/- mice with wild-type mice, two groups found that lack of CD40 has no effect on antigen-induced BALF eosinophilia and airway hyperresponsiveness *in vivo* (14, 16). $CD40L^{-/-}$ mice, on the other hand, show a reduction in BALF eosinophilia compared with wild-type mice in one study (15) but not in another study (16). The latter group also investigated the effect of CD40L deficiency on the development of non-specific hyperresponsiveness *in vivo* and observed that hyperresponsiveness was prevented by the lack of CD40L, in contrast with $CD40^{-/-}$ mice (16). Notwithstanding these conflicting data, however, we conclude that, in the murine models we used, blockade of the CD28/CTLA4:B7-1/B7-2 pathway is much more effective in comparison with the interruption of the CD40L:CD40 pathway.

The extra long-term effect of CTLA4-IgG on the serum levels of OVA-specific IgE in the "mild" model of allergic asthma compared with the more "severe" model exemplifies the qualitative differences besides the quantitative differences between these two murine models. It appears that tolerance induction of the CD4+ T-cell signal transduction pathway or subset involved in B-cell IgE production is differentially regulated under the various conditions of allergen exposure. We believe that the use of diverse sensitization and challenge protocols combined with various mouse strains is valuable because the group of asthma patients is very heterogeneous, both genetically and in the severity of the disease.

The secondary responses group provided some interesting information about the dynamics of the allergic immune response. First, in control-treated mice the magnitude of secondary responses upon OVA aerosol challenge is very similar at three and 14 wk after sensitization, revealing that antigen-specific memory persists for up to 14 wk. Second, re-challenge of control-treated mice does not increase the

magnitude of the tertiary responses compared with those from the secondary response group. This might indicate that the OVA-specific memory T-cell numbers are very similar before aerosol challenge and re-challenge. Alternatively, an increase in memory T-cells upon challenge does not contribute to enhancement of airway manifestations upon re-challenge because the number of OVA-specific memory T-cells induced by systemic sensitization is already sufficient to induce maximal pathological responses. Importantly, the number of eosinophils (and OVA-specific IgE levels) of the secondary responses group is higher compared with those from CTLA4-IgG-treated, OVA-challenged and re-challenged mice. Therefore, this reduction by CTLA4-IgG cannot be ascribed to inhibition of secondary responses resulting in lower tertiary responses.

In summary, this is the first demonstration that in OVA-induced murine models of allergic asthma, CTLA4-IgG induced tolerance to this experimental allergen. Interestingly, tolerance could be induced for airway eosinophilia and OVA-specific IgE but not for non-specific airway hyperresponsiveness, whereas CTLA4-IgG inhibits the development of all of these parameters. This selective tolerization of CD4+ T-cell-dependent effector functions suggests that these effector functions are regulated by different T-cell signal transduction pathways or different CD4+ T-cell subsets in mice (and man) that might differ in their sensitivity to tolerance induction by CTLA4-IgG. Elucidation of the regulation of the various manifestations of allergic asthma will be very helpful in optimizing therapies directed at the allergen-specific T-cell including blockade of co-stimulation. Perhaps the blockade of other T-cell co-stimulatory molecule(s) -*e.g.* ICOS- in combination with CTLA4-Ig is required to completely block T-cell activation in mice during the aerosol challenge period and to induce antigen-specific tolerance to all asthma-like symptoms.

ACKNOWLEDGEMENTS

D.T.D. and E.C.A.M.v.E. are supported by Research Grant 3.2.97.09 from The Netherlands Asthma Foundation. We thank Biogen (Inc., Cambridge, MA, USA) and Genentech (South San Francisco, CA, USA) for kindly providing the hamster antibodies (MR1 and Ha4/8) and murine CTLA4-IgG, respectively. We are grateful to Mirjam Kool and Ilse Bruijstens for expert technical assistance.

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Chapter 6

General summary and discussion

1 Short introduction

Allergic asthma is a chronic inflammatory disorder of the airways characterized by reversible airflow obstruction, persistent airway hyperresponsiveness, chronic airway inflammation and airway remodeling (1). Allergen-specific type 2 T-helper (Th2) cells play a pivotal role in both the initiation and progression of allergic asthma by orchestrating the inflammatory response (2, 3). In animal models of allergic asthma, various experimental treatments have therefore been explored that target the allergen-specific T-cell, including blockade of their co-stimulatory requirements (4, 5).

For optimal activation, CD4+ T-cells require specific antigen recognition by the T-cell receptor (TCR) (signal 1) and additional signals (collectively called signal 2 or co-stimulatory signals), delivered by the same antigen-presenting cell (APC) (6-8). Indeed, antigen binding to the TCR in the absence of co-stimulation not only fails to activate the cell; it also leads to a state of antigen-specific unresponsiveness, called anergy. Such anergic T-cells are refractory to antigen-specific re-stimulation even when the antigen is presented by a professional APC (9-13). In this thesis, the role and therapeutic potential of three main T-cell co-stimulatory pathways in ovalbumin (OVA)-induced murine models of allergic asthma was further investigated (*see* Table 1).

Table1: Overview of T-cell co-stimulatory pathways studied in this thesis

Receptor on T-cell	Ligand(s)	Reviewed in
CD28/CTLA4 (CD152)	B7-1 (CD80)/B7-2 (CD86)	(14-17)
ICOS	B7RP-1 (B7h, GL50, LICOS, B7-H2, ICOSL)	(17-19)
CD40L (CD154)	CD40	(20, 21)

With our studies, we aimed to answer the following questions:

1) What is the role of these T-cell co-stimulatory pathways in the development of Th2-dominated allergic inflammatory responses in mice (paragraph 2)?
2) Asthma patients usually start therapy after the development of airway symptoms while exposure to environmental allergen mostly continues. Therefore, a new murine model of ongoing disease was designed in which treatment starts after development of asthma-like symptoms in mice and antigen exposures are continued (ongoing model). This model was used to investigate the role of two T-cell co-stimulatory pathways in an ongoing response after the development of asthma-like symptoms (paragraph 3).
3) Short-lived treatment that induces selective tolerance to the inhalant allergen would greatly improve the prospects for asthma patients. Not only would they be released from regular intake of medicine but also, more importantly,

the increased risk for infections and cancer due to continuous antigen-specific T-cell suppression would be avoided. Therefore, the long-term effects of blockade of T-cell co-stimulatory pathways during the development of asthma-like symptoms in mice were examined, that is after serum clearance of the blocking agents when mice are re-exposed to the antigen (paragraph 4).

2 Role of T-cell co-stimulatory molecules in the development of Th2-dominated allergic inflammatory responses

2.1.1 The CD28/CTLA4:B7-1/B7-2 pathway

CD28 is the primary co-stimulatory molecule, present on the surface of naïve T-cells. Upon interaction with its ligands B7-1 (CD80) or B7-2 (CD86) on APCs, CD28 tranduces a potent signal that enhances T-cell proliferation and cytokine secretion and sustains the T-cell response.

This research project was based on studies by others and us demonstrating that blockade of the primary T-cell co-stimulatory molecule, CD28, by CTLA4-Ig during antigen sensitization and/or challenge of mice strongly inhibited the development of asthma-like symptoms (22-25). In addition to these blocking studies, the critical role of CD28 signaling in the development of Th2-dominated allergic inflammatory responses in mice has been demonstrated since by gene targeting studies. No asthma-like symptoms could be induced in CD28-deficient ($CD28^{-/-}$) mice (26-28) or B7-1/B7-$2^{-/-}$ mice (29). In all of these studies a decrease in Th2 cytokines in either broncho-alveolar lavage fluid (BALF) or supernatants of antigen re-stimulated lung T-cells *in vitro* was observed. This is in agreement with reports demonstrating the contribution of CD28 signaling to T-cell activation (reviewed in 14-17) and differentiation towards the Th2 phenotype (30-33) and provides a likely explanation for the reduced Th2-dependent asthma-like symptoms in mice by CD28 blockade.

B7-1 and B7-2 appear to support T-cell activation equivalently (34). On most APC populations, B7-2 is expressed constitutively at low levels and is rapidly up-regulated, whereas B7-1 is inducibly expressed later after activation. The earlier expression pattern of B7-2 suggests that B7-2 is the more important co-stimulator for initiating T-cell responses. Blockade of B7-2 by monoclonal antibody (mAb) administration has consistently been shown to inhibit allergic airway manifestations in mice although to a variable degree (24, 35-37). In contrast, the contribution of B7-1 to the development of these manifestations is more controversial. In some studies it was demonstrated that treatment of mice with anti-B7-1 mAbs (24, 35) or a mutated form of CTLA4-Ig that selectively binds to B7-1 (38) had no effect (37) or only (partially) diminished airway eosinophilia (24, 35). Other studies supported a partial but similar role for B7-1 and B7-2 in the development of airway hyper-

responsiveness and serum IgE as well (26, 36). Moreover, comparison of mice deficient in B7-1 or B7-2 demonstrated that these molecules have complementary roles in the development of Th2-dominated allergic inflammatory responses (29). Some factors that may account for the diverse results include different protocols, mouse strains, doses and kinetics of inhibitors and receptor blockade versus active signaling.

With regard to peripheral blood T-cells derived from atopic asthmatics, B7-2 appears to be the principal co-stimulatory ligand for allergen-induced proliferation and cytokine production (39, 40). However, resident allergen-specific T-cells in asthmatic bronchial tissue were shown to require both B7-1 and B7-2 ligation for allergen-induced cytokine production (40). These differential requirements for B7-1 and B7-2 engagement observed in bronchial tissue and blood may reflect differences between the two sites in the availability of B7-1.

The second receptor for the B7-1 and B7-2 ligands, CTLA4 (CD152), is expressed on activated T-cells and delivers an inhibitory signal to terminate the T-cell response. CTLA4 not only inhibits T-cell proliferation (41-43), but also has been implicated in Th2 differentiation. $CTLA4^{-/-}$ CD4+ T-cells demonstrate a Th2 cytokine profile (44, 45) and naïve CD4+ T-cells show a Th1 cytokine profile after CTLA4 ligation *in vitro* (46) and *in vivo* (47). Together, this would mean that blockade of CTLA4 aggravates Th2-dominated asthma manifestations in mice. Indeed, administration of an anti-CTLA4 mAb during OVA sensitization of BALB/c mice resulted in an enhanced Th2 priming *in vivo* and an elevation of asthma-like symptoms following aerosol challenge compared with control-treated mice (48). In addition, a marked reduction in transforming growth factor (TGF)-β was observed both *in vitro* and *in vivo*. Because CTLA4 has been described to induce TGF-β production by murine CD4+ cells (49) and TGF-β in turn has been demonstrated to inhibit airway eosinophilia (50, 51) and airway hyperresponsiveness (51, 52), this might offer an explanation for the increased Th2-dominated allergic inflammatory responses in mice treated with anti-CTLA4 mAb. In contrast with BALB/c mice, Th2 priming, asthma manifestations and TGF-β production were not affected in C57BL/6 mice (48) that are genetically less prone to Th2 responses. In accordance herewith, it has been reported that CTLA4 engagement does not to regulate TGF-β production in T-cells of C57BL/6 mice (53). Lastly, when anti-CTLA4 mAb was administered solely during challenge of OVA-sensitized BALB/c mice the asthma phenotype was unaltered (48). This latter finding was corroborated in a similar study using the same mAb performed in our laboratory (D.T. Deurloo and E.C.A.M. van Esch, unpublished observations) and suggests that memory T-cells are refractory to CTLA4-mediated inhibition of allergic responses.

2.1.2 A putative additional B7-1/B7-2 receptor

Since the unraveling of the human genome, the CD28 and B7 families are rapidly growing (reviewed in 17, 18, 54, 55). Moreover, generation of mice lacking both CD28 and CTLA-4 has produced evidence for a third B7-1/B7-2 receptor (56). In this study, CD28/CTLA4$^{-/-}$ CD4+ T-cells demonstrated considerable residual B7-1/B7-2-dependent proliferation upon polyclonal stimulation *in vitro*. In addition, T-cell co-stimulation *via* this putative B7-1/B7-2 receptor was shown to contribute substantially to Th1-mediated cardiac allograft rejection *in vivo*, in contrast with long-term allograft survival in B7-1/B7-2$^{-/-}$ mice (57, 58).

Although no manifestations of allergic asthma can be induced in CD28$^{-/-}$ mice (26-28), the presence of lymphocytes and interferon-γ in the BALF (26, 27) and the priming of lymphocytes in the lungs, spleen and lymph nodes (26-28) is observed in these mice. These data on residual T-cell activation in CD28$^{-/-}$ mice but not in B7-1/B7-2$^{-/-}$ mice (29) suggested a potential role for the putative B7-1/B7-2 receptor. Co-stimulation by the supposed B7-1/B7-2 receptor would then result in allergen-specific T-cell activation that is not strong enough to induce the asthma-like symptoms. However, the failure to induce these symptoms in CD28$^{-/-}$ mice might also be due to the presence of CTLA4 that can signal negatively on its own (59-61), thus opposing potential T-cell co-stimulation *via* the putative B7-1/B7-2 receptor. So, unmasking the possible role of co-stimulation by the supposed B7-1/B7-2 receptor in T-cell activation required the absence of both CD28 and CTLA4.

Therefore, we investigated if T-cell co-stimulation *via* the putative B7-1/B7-2 receptor contributes to the induction of Th2-dominated model of allergic asthma using the novel CD28/CTLA4$^{-/-}$ mouse strain (chapter 2). In agreement with an earlier study by Mark *et al*. (29), we failed to induce any of these asthma-like symptoms in the B7-1/B7-2$^{-/-}$ mice. Interestingly, also CD28/CTLA4$^{-/-}$ mice showed no manifestations of allergic asthma upon sensitization and challenge with OVA. Moreover, we were unable to demonstrate any sign of residual T-cell activation in both the thoracic lymph nodes and lungs from CD28/CTLA4$^{-/-}$ mice upon OVA-sensitization and challenge *in vivo*.

So, T-cell co-stimulation *via* the putative B7-1/B7-2 receptor appears to have no role in the induction of Th2-mediated asthma-like symptoms in the murine model that we used. Conversely, further evidence is provided for the critical role of CD28 signaling in the development of Th2 responses (26-28, 32).

However, the prerequisite of CD28-mediated T-cell co-stimulation for the induction of asthma-like symptoms in mice does not exclude important contributions by other co-stimulatory molecules at various stages in the (re-)activation of T-cells. Therefore, the effects of blockade of two other well-known receptor-ligand pairs of T-cell co-stimulation, the ICOS:B7RP-1 and CD40L:CD40 pathways in the development of Th2-dominated allergic inflammatory responses were studied in this thesis.

2.2 The ICOS:B7RP-1 pathway

Inducible co-stimulator (ICOS) is a recently discovered CD28 family member that binds its own B7 family member, B7 related protein 1 (B7RP-1) (also known as B7h, B7-H2, GL50 or ICOSL or LICOS) (reviewed in 17-19). ICOS is induced rapidly on T-cells after TCR engagement and is retained on many memory T-cells, suggesting that ICOS provides co-stimulatory signals to activated T-cells. Indeed, during the initial activation of naïve T-cells, the effects of ICOS on T-cell proliferation and interleukin (IL)-2 production are modest in comparison to those of CD28. Signals through ICOS seem to be more important for the regulation of cytokine production by effector T-cells.

With regard to the role of ICOS in the development of asthma-like symptoms, it has been shown that sensitization and aerosol challenge of $ICOS^{-/-}$ mice with OVA resulted in strongly inhibited serum OVA-specific IgE levels compared with wild-type mice (62). However, $ICOS^{-/-}$ mice exhibited similar numbers of eosinophils, lymphocytes and monocytes/macrophages as wild-type mice. In contrast, in a study using a murine model of allergic asthma induced by the *Schistosoma mansoni* parasite, it was demonstrated that administration of ICOS-Ig during sensitization and challenge of C57BL/6 mice resulted in a reduced number of inflammatory cells in the BALF accompanied by a decrease in the level of IL-5 (63), but no effect on the serum total IgE level was observed.

In chapter 4, it is reported that treatment of mice with hICOS-Fc throughout the aerosol challenge period significantly reduced the number of inflammatory cells in the BALF compared with control-treated animals, but no changes in airway hyperresponsiveness *in vivo* and serum OVA-specific IgE levels were observed. Whereas we used OVA as antigen and BALB/c mice, our results are thus very much in agreement with those of Tesciuba *et al*. (63). The mechanism responsible for the reduced amount of BALF inflammatory cells by ICOS blockade observed in both studies is unknown at present but is not due to a lack of T-cell priming nor to a defect in Th2 differentiation as determined by unaltered proliferation and Th2 cytokine production.

To date, no studies on the role of ICOS in human asthma have been reported.

2.3 The CD40L:CD40 pathway

The CD40 ligand (CD40L; CD154):CD40 pair of counter-receptors (reviewed in 20, 21) is the best-characterized pathway of T-cell co-stimulation from the tumor necrosis factor (TNF) receptor-ligand families (reviewed in 64-66).

CD40L is transiently expressed on activated CD4+ T-cells that interacts with CD40 found on many cell types, including endothelial cells, fibroblasts, epithelial cells, mast cells and professional APCs. In addition to evidence for direct T-cell co-stimulation *via* CD40L (67-72), triggering of CD40 contributes indirectly to T-cell

co-stimulation by the up-regulation of B7-1 and B7-2 expression on APCs, thus enhancing T-cell activation through CD28-mediated signaling (73, 74).

The importance of the CD40L:CD40 pathway in the induction of asthma-like symptoms has previously only been investigated in mice deficient in either CD40L or CD40 (75-77). Because ligation of CD40 on B cells, together with IL-4 (78), is critical for their activation and the induction of IgE isotype switching (79), the absence of (allergen-specific) IgE in serum was anticipated and demonstrated in all of these studies. Comparing $CD40^{-/-}$ mice with wild-type mice, two groups found that lack of CD40 has no effect on antigen-induced BALF eosinophilia and airway hyperresponsiveness *in vivo* (75, 77). $CD40L^{-/-}$ mice, on the other hand, show a reduction in BALF eosinophilia compared with wild-type mice in one study (76) but not in another study (77). The latter group also investigated the effect of CD40L deficiency on the development of non-specific hyperresponsiveness *in vivo* and observed that hyperresponsiveness was prevented by the lack of CD40L, in contrast with $CD40^{-/-}$ mice (77).

In the study described in chapter 5, we described the effects of administration of the anti-CD40L mAb MR1 during OVA-challenge of sensitized mice on the development of asthma-like symptoms. Anti-CD40L mAb prevented the up-regulation of serum OVA-specific IgE levels, thus clearly demonstrating the success of this treatment, but did not alter the degree of OVA-induced airway hyperresponsiveness *in vivo* and BALF eosinophilia. Moreover, when administered in the presence of CTLA4-IgG, anti-CD40L mAb did not add to the inhibitory effects of CTLA4-IgG in the model. Recently, very similar results were obtained with the same anti-CD40L mAb in a murine model of allergic asthma when administered during OVA-sensitization or challenge (80).

The levels of Th2 cytokines produced by bronchial tissue or peripheral blood T-cells from asthmatics in response to allergen were not affected by the interruption of the CD40L:CD40 pathway (40). In fact, it has been shown that CD40 signals delivered by alveolar macrophages can suppress IL-5 production by peripheral blood CD4+ cells in an allergen-stimulated co-culture system. Alveolar macrophages derived from patients with atopic asthma were shown to express lower levels of CD40 that was related to lower IL-12 production by alveolar macrophages and higher IL-5 production by CD4+ T-cells (81).

So, all of the three pathways of T-cell co-stimulation studied contribute to the development of Th2-dominated allergic inflammatory responses in mice. Blockade of the CD40L:CD40 interaction prevented the up-regulation of antigen-specific IgE, whereas hICOS-Fc administration reduced the total number of inflammatory cells in the BALF. However, blocking- and gene targeting studies demonstrated that only the CD28/CTLA4:B7-1/B7-2 pathway is essential for the development of all asthma-like symptoms in mice.

3 Role of T-cell co-stimulatory molecules in ongoing Th2-dominated allergic inflammatory responses

In contrast to the experimental studies that investigated the role of T-cell co-stimulatory molecules in the development of Th2-mediated asthma-like symptoms in mice, asthmatic patients start therapy after the development of airway symptoms, while exposure to environmental allergen mostly continues.

3.1 The CD28/CTLA4:B7-1/B7-2 pathway

Notably, studies *in vitro* have demonstrated that effector and memory T-cells are less dependent on CD28-mediated co-stimulation in comparison with naïve T-cells (31, 82, 83), whereas studies *in vivo* on the effectiveness of giving CTLA4-Ig after priming of immune responses have yielded conflicting results (84).

In the study described in chapter 3, it was investigated whether CTLA4-IgG can reverse established asthma manifestations in a novel murine model of ongoing disease, in which therapy starts after the development of asthma-like symptoms while OVA exposures are continued. In OVA-sensitized mice, airway manifestations were induced by a first series of aerosol challenges. CTLA4-IgG was then administered followed directly by a second series of aerosols. Thus, at the time of CTLA4-IgG administration, not only naïve but also antigen-experienced (memory and effectors) OVA-specific T-cells are present that may be regulated by other co-stimulatory molecules. In this ongoing murine model of allergic asthma -that was validated by the "gold-standard" corticosteroid dexamethasone- CTLA4-IgG was still effective (85). However, airway eosinophilia and OVA-specific IgE levels could only be partially inhibited and airway hyperresponsiveness was unaffected. CTLA4-IgG may have inhibited antigen-experienced OVA-specific T-cells in this model. In agreement herewith, CTLA4-Ig was shown to inhibit allergen-induced cytokine production by resident T-cells in asthmatic bronchial tissue derived from mild atopic asthmatics (86). In addition, allergen-induced proliferation of BALF T-cells from atopic subjects was inhibited by anti-B7-2 mAb (39).

In addition, when newly recruited- or resident naïve CD4+ T-cells that were not previously activated, contribute to disease, the effectiveness of CTLA4-IgG may be due to its inhibition of the activation of these cells. T-cells develop from a common lymphoid progenitor in the bone marrow that also gives rise to B-cells, but those progeny destined to give rise to T-cells leave the bone marrow and migrate to the thymus. This is the reason they are called thymus-dependent (T) lymphocytes or T-cells. With increasing age, the lymphatic thymic mass decreases and thymocyte production correspondingly declines (thymic involution) (87). Interestingly, recent data suggest, however, that the adult thymus can remain active even late in life supplying functional T-cells to the periphery (called recent thymic emigrants) in humans (88, 89), sheep (90) and mice (91, 92). On the one hand, it will be of interest

to determine the contribution of these recent thymic emigrants to T-cell-mediated diseases, including allergic asthma, in people already suffering from full-blown disease. On the other hand, it will be important to resolve the life span of effector and memory antigen-specific T-cells. From animal studies, it is concluded that the average life span of a memory T-cell is determined by how frequently the antigen is encountered, as well as by how much space is available in the T-cell compartment (93). Several recent reports have confirmed the presence of antigen-specific memory T-cells in non-lymphoid compartments long after priming in mice (94, 95). Future studies are needed to clarify whether CTLA4-IgG mediated its inhibitory effect on recent thymic emigrants and/or is able to inhibit antigen-experienced T-cells.

3.2 The ICOS:B7RP-1 pathway

In the ongoing murine model of allergic asthma, the partial inhibition by CTLA4-IgG may be due to the predominance of antigen-experienced- over newly recruited- or resident naïve OVA-specific T-cells at the time of treatment. Other co-stimulatory molecules may regulate these antigen-experienced T-cells.

Interestingly, two studies of Coyle and colleagues provided evidence that ICOS co-stimulation might contribute more to the effector phase than to the initiation of Th2-dominated allergic inflammatory responses (96, 97). In the first study, ICOS blockade during antigen challenge reduced lung inflammation and non-specific airway hyperresponsiveness following adoptive transfer of highly polarized Th2 effector populations to naïve mice (96). In the second study, administration of mAb 12A8 against ICOS solely during the last (seventh) aerosol challenge of OVA-sensitized mice significantly reduced the amount of BALF inflammatory cells and airway hyperresponsiveness, whereas ICOS blockade only at the time of sensitization had little effect upon subsequent airway challenge (97). Therefore, in the study described in chapter 4, we investigated the effects of ICOS blockade by ICOS-Fc in the ongoing murine model of allergic asthma. Surprisingly, in the ongoing model, administration of ICOS-Fc had no effect on any of the final asthma-like symptoms. Likewise, ICOS-Fc did not inhibit IL-5 production by primed lung T-cells upon OVA re-stimulation *ex vivo*. When we administered the same dose of the 12A8 mAb in the murine model of ongoing disease, also no alteration of the final manifestations of allergic asthma was observed. In addition, the combined administration of CTLA4-IgG and mAb 12A8 against ICOS did not have a synergistic effect in the ongoing murine model of allergic asthma. The discrepancies between the results of Coyle and colleagues and our results might be explained by the use of Th2 cells that were generated *in vitro* and may result in effector T-cells with migratory and functional properties that are uncommon *in vivo* (96) or differences in the time point of measurement of the various parameters after the last aerosol challenge, the sensitization and challenge protocol and the strain of mouse used (Ref 97 versus chapter 4).

So, in an ongoing murine model of allergic asthma, only CTLA4-IgG was effective in that it could partially inhibit airway eosinophilia and the OVA-specific IgE levels (85), whereas ICOS-Fc had no effect on any of the final asthma-like symptoms. Future studies using the ongoing model may elucidate which (combination of) other signals are required for allergic inflammatory responses dominated by antigen-experienced CD4+ T-cells.

4 Long-term effects of blockade of T-cell co-stimulatory molecules

Studies *in vitro* have demonstrated that in the absence of co-stimulation, antigen-stimulated T-cells besides being not optimally activated are also rendered anergic that is, unresponsive to antigenic re-stimulation in the presence of co-stimulation (9-13). *In vivo*, blockade of T-cell co-stimulation around the time of engraftment was shown to allow for long-term allograft survival in several transplantation models (*see* 98). Short-lived treatment that induces selective tolerance to the inhalant allergen would greatly improve the prospects for asthma patients. Not only would they be released from regular intake of medicine but also more importantly, the increased risk for infections and cancer due to continuous antigen-specific T-cell suppression would be avoided. However, the long-term effects of T-cell co-stimulation blockade during the development of antigen-induced asthma-like symptoms in mice had not been studied so far.

4.1 The CD28/CTLA4:B7-1/B7-2 pathway

In the study described in chapter 5, it was investigated if blockade of the CD28/CTLA4:B7-1/B7-2 pathway during the aerosol challenge period of antigen-sensitized mice results in long-term amelioration of asthma-like symptoms that is, not only immediately after challenge but also upon antigen re-challenge after serum clearance of experimental treatment, eleven weeks later. Interestingly, CTLA4-IgG could induce tolerance for airway eosinophilia and OVA-specific IgE but not for non-specific airway hyperresponsiveness. So, whereas CTLA4-Ig inhibits the development of all of these manifestations of allergic asthma (22-25, 99) as described above in paragraph 2.1, the tolerization of these parameters was dissociated in this study. Perhaps, CTLA4-IgG had selectively tolerized B7-1/B7-2-dependent T-cell signal transduction pathways. Alternatively, the various manifestations of allergic asthma are controlled by different subsets of (allergen-specific) CD4+ T-cells that might differ in their sensitivity to tolerance induction by CTLA4-IgG. Notably, Campbell *et al*. identified specialized “B helper” and “tissue inflammatory” CD4+ T-cell effector subsets that developed concurrently from common naïve precursors during the primary immune response in BALB/c mice (100). Similarly, two distinct types of human CD4+ and CD8+ memory cells have been identified: “central memory” cells present in secondary lymphoid tissues and “effector memory” cells residing in peripheral tissues that have

been suggested to perform different functions upon antigenic re-encounter (*see* 101). Elucidation of the regulation of the various manifestations of allergic asthma will be very helpful in optimizing therapies directed at the allergen-specific T-cell including blockade of co-stimulation.

4.2 The CD40L:CD40 pathway

In vitro, it was found that the successful induction of unresponsiveness toward soluble antigens with CTLA4-Ig needed complete blockade of antigen-induced proliferation during the induction culture (102). Along this line, although CTLA4-IgG greatly reduced the asthma-like symptoms in mice, the symptoms were not fully blocked suggesting that not all OVA-specific CD4+ T-cells were inhibited and hence (selectively) tolerized. These non-tolerized antigen-specific T-cells are likely to be responsible for the residual airway eosinophilia and OVA-IgE levels observed upon OVA re-challenge. Interestingly, a number of studies *in vitro* and *in vivo* on the induction of antigen-specific tolerance have demonstrated that blockade of both the CD28/CTLA4:B7-1/B7-2 and the CD40L:CD40 pathway is more effective than blockade of either pathway alone (103-108). Therefore, the immediate and long-term effects of blockade of T-cell co-stimulation by the anti-CD40L mAb MR1, CTLA4-IgG or the combination of these treatments during the aerosol challenge period of antigen-sensitized mice were compared. However, anti-CD40L mAb had not a long-term effect by itself. Moreover, in combination with CTLA4-IgG, anti-CD40L mAb did not augment the CTLA4-IgG-mediated inhibition of asthma-like symptoms (as discussed above in paragraph 2.3) or the tolerance induced by CTLA4-IgG. Perhaps the blockade of yet other T-cell co-stimulatory molecule(s) in combination with CTLA4-Ig is required to completely block T-cell activation in mice during the aerosol challenge period and to induce antigen-specific tolerance to all manifestations of allergic asthma. Administration of anti-ICOS mAb 12A8 alone or in combination with CTLA4-Ig during the challenge period of OVA-sensitized mice demonstrated that this mAb had no long-term effect by itself or adds to the tolerance induced by CTLA4-IgG (D.T. Deurloo, unpublished observations).

So, CTLA4-IgG could induce partial tolerance in an OVA-induced murine model of allergic asthma for BALF eosinophilia and serum OVA-specific IgE levels. In contrast, mAbs against CD40L or ICOS appeared to have no long-term effect upon OVA re-challenge of mice after serum clearance in our models.

5 T-cell co-stimulatory molecules and regulatory T-cells

In our studies, T-cell co-stimulation was blocked with the purpose of inhibiting allergen-specific Th2 cells. Interestingly, in recent years T-cell co-stimulatory receptors have also been found on the surface of so-called regulatory T-cells, a subset of lymphocytes that specifically suppresses immune responses (*see* 109, 110). Some

T-cells that have suppressor activity are part of a unique lineage of CD4+ T-cells that are "naturally occurring" and are present in the thymus and peripheral lymphoid tissues of mice and humans (reviewed in 110-112). The "naturally occurring" suppressor T-cell population can be identified in naïve animals by expression of the CD25 antigen. CD4+CD25+ T-cells are potent suppressors of the activation of both CD4+ and CD8+ T-cells *in vitro*. Transfer of BALB/c splenic cell suspensions eliminated of CD4+CD25+ cells to syngeneic T-cell deficient mice has been shown to induce various auto-immune diseases (113), demonstrating that these cells provide an additional mechanism for peripheral tolerance besides anergy. The suppression of these CD4+CD25+ cells might be mediated by both suppressor cytokines - such as IL-4, IL-10 and TGF-β - and cell-contact-dependent mechanisms (110-112).

CD28 has been shown to control the development and homeostasis of CD4+CD25+ T-cells (114), but is dispensable for their suppressive function *in vitro* (115) and *in vivo* (116). In fact, CD4+CD25+ regulatory T-cells were shown to display an anergic phenotype *in vitro*, and CD28 co-stimulation of regulatory T-cells reverses their anergic phenotype and abolishes their suppressive capabilities (15, 117). Another feature of CD4+ CD25+ regulatory T-cells is their constitutive expression of CTLA4 (115). However, the potential functional role of CTLA4 in CD4+CD25+ T-cell function remains controversial (110-112).

In one study, it was determined whether CD4+CD25+ T-cells down-regulate Th2 cell-mediated OVA-induced eosinophil recruitment in the airways (118). BALB/c Rag-2-/- mice -which lack mature T- and B-cells- received CD4+CD25+ T-cell-depleted splenocytes or unfractioned splenocytes from non-immunized OVA-specific TCR transgenic mice. However, the depletion of CD4+CD25+ T-cells reduced the number of eosinophils and levels of IL-4 and IL-5 in the BALF upon sensitization and challenge with OVA, whereas an increase in neutrophils, characteristic for Th1-mediated inflammation (119), was observed. In agreement herewith, the depletion of CD4+CD25+ T-cells prevented antigen-induced Th2 cell differentiation *in vitro* but increased the differentiation of Th1 cells. So, this study suggests that CD4+CD25+ T-cells modulate T helper differentiation towards the Th2 phenotype and thus up-regulate Th2 cell-mediated allergic inflammation in the airways.

Other types of suppressor T-cells can be induced from conventional CD4+ T-cells by specific experimental manipulations *in vitro* that produce predominantly TGF-β or IL-10 (designated T helper 3 (120) or T regulatory 1 (121), respectively). *In vivo,* intranasal exposure to OVA has been shown to result in the induction of CD4+ regulatory T-cells -producing primarily IL-10 and low levels of IL-4 (121) - that inhibited the development of asthma manifestations upon subsequent re-challenge of mice with OVA (122, 123). Interestingly, both the development and inhibitory function of CD4+ regulatory T-cells were shown to be dependent on the ICOS:B7RP-1 signaling pathway (123).

Although it is unclear if the various regulatory T-cells play a role in the murine models of allergic asthma that we used, the functional consequences of CTLA4-IgG or ICOS-Fc administration in our studies may have been influenced by their effects not only on Th2 cells but also on regulatory T-cells. Possibly, the specific targeting of regulatory T-cell function is an interesting strategy in the treatment of allergic diseases.

6 Clinical implications from our studies

Murine models of allergic asthma have been very valuable in unraveling the underlying cellular and molecular mechanisms. The concept of the role of T-cells, their heterogeneity, and specialized functions all first emerged in the mouse and subsequently these findings were confirmed in humans (124, 125).

However, some of the experimental therapies that were very effective in mice had limited benefit in humans (126, 127). Nevertheless, at this place, we would like to extrapolate the results from our studies with great care to the human situation. Notably, some experiments were performed in both a “mild” and a more “severe” murine model of allergic asthma as a representation of the varied disease severity that is observed among asthma patients as well. In addition, in some of our studies we also made use of an ongoing murine model of allergic asthma in an attempt to more closely resemble the clinical situation.

Based on the essential role of the CD28/CTLA4:B7-1/B7-2 pathway in the development of Th2-dominated allergic inflammatory responses (chapter 2), blockade of this pathway may be an effective alternative therapy for asthma patients that are in clinical remission. For example, people suffering from seasonal allergies or patients that avoided the allergen for a long period of time. This prediction is done with the assumption that the number of antigen-experienced cells has severely declined in these cases. Because CTLA4-IgG could induce partial tolerance (chapter 5), these patients might then even develop tolerance to the inhalant allergen, thus experiencing long-lasting improvement of their clinical situation.

For asthma patients with ongoing disease (*e.g.* people allergic to common allergens such as the excrements of house dust mite), blockade of T-cell co-stimulation by CTLA4-Ig may not be sufficient. The level of serum OVA-specific IgE and BALF eosinophilia were only partially reduced by CTLA4-IgG administration in the ongoing murine model of allergic asthma (chapter 3). Especially patients with more severe ongoing disease may require additional anti-inflammatory drugs as a clear difference in the effectiveness of CTLA4-Ig between the “mild” and more “severe” model was observed. For asthma patients with ongoing disease, CTLA4-Ig might serve as an “add-on” therapy to reduce the potential side effects by inhaled corticosteroids when used in escalating doses. Moreover, up to 25% of patients with severe asthma show poor clinical responses to high doses of glucocorticosteroids (128).

Blockade of the other pathways of T-cell co-stimulation studied in this thesis, the ICOS:B7RP-1 and CD40L:CD40 pathways, was in all studies less effective compared with CTLA4-IgG. So, CTLA4-Ig appears to have the greatest therapeutic potential.

7 Possible future directions for research

Concerning the regulation of asthma-like symptoms in mice, it will be of interest to determine:

- the contribution of recent thymic emigrants to T-cell mediated diseases, including allergic asthma, in mice already suffering from full-blown disease,
- the life-span of antigen-specific effector and memory T-cells,
- the specific functions of T-cell subsets present in lymphoid and peripheral tissues,
- the activation requirements of T-cell signaling pathways or T-cell subsets involved in the various manifestations of allergic asthma in mice,
- the role and regulation of the various regulatory T-cells.

Concerning T-cell co-stimulation, it will be of interest to determine:

- the expression and function of the last to be discovered members of the expanding families of T-cell co-stimulatory receptors and their ligands,
- the unique and overlapping biochemical signals and functional responses of the individual members of the T-cell co-stimulatory receptor families,
- how the signals from the various T-cell co-stimulatory and inhibitory receptors are integrated,
- which interactions are critical at the various stages of the immune responses,
- the role of localized T-cell co-stimulation in peripheral tissues,
- which immuno(patho)logical responses are influenced by which T-cell co-stimulatory pathways.

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LIST OF ABBREVIATIONS

–/–	homozygous deficiency
AHR	airway hyper-responsiveness
Alum	aluminiumhydroxide
APC	antigen presenting cell
BAL(F)	bronchoalveolar lavage (fluid)
BSA	bovine serum albumin
CD	cluster of differentiation
CD80	B7-1
CD86	B7-2
CD152	CTLA4
CD154	CD40 ligand
CD40L	CD40 ligand
cDNA	complementary Deoxyribo Nucleic Acid
CHO cells	Chinese Hamster Ovary cells
CTLA4	Cytotoxic T Lymphocyte-associated Antigen 4
d	days
DC	dendritic cell
DIG	digoxigenin
DRC	dose-response curve
EDTA	ethylene diamine-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
eo	eosinophils
EU	experimental units
Fab	antigen-binding fragment
FACS	fluorescence-activated cell sorter
Fc	constant region
FcR	Fc receptors (*e.g.*, FcγRI)
FCS	fetal calf serum
FITC	fluorescin isothiocyanate
h	hours/human
ham	hamster
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
L	ligand
LN	lymph node
m	murine
mAb	monoclonal antibody
MHC-II	major histocompatibility complex class II
MNC	mononuclear cells
mo	months
mRNA	messenger ribonucleic acid
n	number of subjects
neutro	neutrophils
OVA	ovalbumin
p	probability value
PBS	phosphate-buffered saline
PD	programmed death
Penh	enhanced pause
R	receptor
SEM	standard error of the mean
STAT	signal transducer and activator of transcription
TCR	T-cell receptor
Th cell	T helper cell
TLN	thoracic lymph nodes
wk	weeks

SAMENVATTING IN HET NEDERLANDS

Allergisch astma

Allergisch astma is een veel voorkomende ziekte waarbij mensen benauwd worden wanneer ze de stof inademen waar ze allergisch voor zijn geworden. Daarnaast trekken de spieren in de luchtwegen van astmapatiënten zich sterker samen bij koude lucht, sigarettenrook en luchtwegvernauwende stoffen zoals histamine en methacholine. Dit wordt aspecifieke luchtweghyperreactiviteit genoemd.

Het immuunsysteem (de afweer) dat het lichaam beschermt tegen schadelijke indringers zoals virussen en bacteriën, speelt ook een cruciale rol bij allergisch astma. Bij mensen die allergisch zijn, treedt er namelijk een overgevoeligheidsreactie op van het immuunsysteem voor bepaalde stoffen, de zogenaamde allergenen. De allergische reactie kan plaatsvinden in de neus (rhinitis), in de longen (astma), in de darmen (voedselallergie) of in de huid (eczeem).

Het immuunsysteem bestaat uit vele soorten cellen met elk een specifieke taak. Als het allergeen in de slijmvliezen van de longen komt, wordt het opgenomen door antigeen-presenterende cellen. Deze cellen "knippen" het allergeen in stukjes en brengen deze deeltjes in de lymfknopen in contact met zogenaamde T-cellen. De miljoenen T-cellen die in het lichaam aanwezig zijn verschillen van elkaar omdat ze allemaal een andere T-cel receptor hebben die elk een specifiek stukje eiwit kan herkennen. Er zijn ook enkele allergeen-specifieke T-cellen die elk een ander stukje van het allergeen kunnen herkennen. Na herkenning van het stukje allergeen, vermenigvuldigen deze T-cellen zich door deling en scheiden ze zogenaamde cytokinen uit (zie figuur 1 op pagina 9). Deze cytokinen kunnen andere typen immuuncellen bij de allergische reactie betrekken. Het cytokine interleukine-4 dat door de allergeen-specifieke T-cel geproduceerd wordt, zorgt er voor dat B-cellen immunoglobuline E (IgE) tegen het allergeen gaan maken. Het allergeen-specieke IgE bindt aan receptoren op het oppervlak van mestcellen. Dit wordt sensibilisatie genoemd. Wanneer het allergeen opnieuw in de luchtwegen komt (provocatie) en aan het allergeen-specieke IgE op de mestcel bindt, worden de mestcellen gestimuleerd en geven hun luchtwegvernauwende stoffen en ontstekingsmediatoren vrij. Een ander cytokine, interleukine-5, betrekt eosinofiele granulocyten (eosinofielen) bij de allergische reactie. Eosinofielen kunnen ook ontstekingsmediatoren uitscheiden. Het cytokine interleukine-13 speelt een belangrijke rol bij het ontstaan van luchtweghyperreactiviteit en bij de overmatige slijmproductie.

Naast de aanvallen van benauwdheid is de voortdurende aanwezigheid van verhoogde aantallen T-cellen, eosinofielen en diverse andere immuuncellen (chronische ontsteking) in de longen een belangrijk kenmerk van allergisch astma.

Tengevolge van deze chronische ontsteking verandert de structuur van het longweefsel. Hierdoor vermindert de longfunctie van astmapatiënten in de loop der jaren.

T-cel co-stimulatie
Voor activatie van de T-cel is niet alleen herkenning van een stukje eiwit door de T-cel receptor nodig, maar ook aspecifieke, zogenaamde co-stimulatie signalen. Antigeen-presenterende cellen die een virus, bacterie of allergeen hebben opgenomen, brengen op hun oppervlak moleculen (liganden) tot expressie die aan co-stimulatie receptoren op de T-cel kunnen binden. Omdat voor volledige activatie van de T-cel beide signalen nodig zijn, kan een stukje eiwit van het lichaam zelf een T-cel niet activeren aangezien de co-stimulatie signalen ontbreken. Zonder co-stimulatie zal een T-cel zich niet delen en geen cytokinen produceren.

Muizenmodellen voor allergisch astma
Om mechanismen die leiden tot allergisch astma te onderzoeken en om nieuwe medicijnen te ontwikkelen, kunnen symptomen van astma in de muis geïnduceerd worden. Voor de experimenten beschreven in dit proefschrift werd het eiwit ovalbumine als modelallergeen gebruikt. De dieren krijgen eerst een injectie met ovalbumine in de buikholte om ze te sensibiliseren. Als de muizen vervolgens na enige tijd een vernevelde oplossing van ovalbumine inademen (provocatie) ontstaat er een ontstekingsreactie in de longen met symptomen die ook bij astmapatiënten aangetroffen worden. Hiertoe behoren luchtweghyperreactiviteit voor methacholine en de infiltratie van ontstekingscellen, met name eosinofielen, in de longen. Daarnaast wordt een grote hoeveelheid ovalbumine-specifiek IgE in het bloed gevonden.

Doel van dit proefschrift
De huidige therapie voor astmapatiënten bestaat uit medicijnen die de luchtwegen verwijden bij een astma-aanval en uit ontstekingsremmers, voornamelijk glucocorticosteroïden. Deze corticosteroïden geven bij hoge dosis echter veel bijwerkingen. Omdat allergeen-specifieke T-cellen een centrale rol spelen bij het ontstaan en de voortgang van allergisch astma, zou het remmen van alleen deze T-cellen een goede therapie kunnen zijn.

In dit proefschrift is de rol van verschillende T-cel co-stimulatie moleculen in diverse muizenmodellen voor allergisch astma onderzocht. Dit om te onderzoeken of blokkade van deze moleculen een nieuwe behandeling voor allergisch astma zou kunnen zijn. Bovendien is onderzocht of de T-cellen ook enkele weken na blokkade nog inactief zijn en als gevolg daarvan geen kenmerken van astma meer kunnen veroorzaken.

Hoofdstuk 2

Het belangrijkste co-stimulatie molecuul op de T-cel is CD28. Er zijn twee liganden voor deze receptor op antigeen-presenterende cellen, B7-1 en B7-2. Deze liganden binden niet alleen aan CD28 maar ook aan een tweede receptor op de T-cel, CTLA4 (zie figuur 2 op pagina 13). CTLA4 komt alleen op het oppervlak van geactiveerde T-cellen voor en geeft in tegenstelling tot CD28 een remmend signaal aan de T-cel.

Naast de CD28 en CTLA4 receptoren is er mogelijk nog een derde receptor voor de B7 liganden. Dit bleek uit experimenten waarin muizen die door een verandering in hun genen geen CD28 en CTLA4 moleculen hebben (CD28/CTLA4$^{-/-}$ muizen) werden vergeleken met muizen die de B7 liganden missen (B7-1/B7-2$^{-/-}$ muizen). In CD28/CTLA4$^{-/-}$ muizen vond een T-cel-afhankelijke afstoting van een transplantaat plaats, terwijl B7-1/B7-2$^{-/-}$ muizen het transplantaat accepteerden. In andere ziekten die door T-cellen worden veroorzaakt zoals allergisch astma was de rol van deze veronderstelde B7 receptor nog onbekend.

In de studie die wordt beschreven in hoofdstuk 2 van dit proefschrift is daarom onderzocht of T-cel co-stimulatie via deze veronderstelde receptor bijdraagt aan het ontstaan van astma symptomen in de muis. Het bleek echter dat zowel in CD28/CTLA4$^{-/-}$ muizen als in B7-1/B7-2$^{-/-}$ muizen geen enkel astmasymptoom met ovalbumine geïnduceerd kon worden. De veronderstelde receptor speelt dus geen rol bij het ontstaan van astma symptomen in ons diermodel. Gebleken is echter dat CD28 hiervoor cruciaal is.

Hoofdstuk 3

Om de interactie tussen de B7 liganden en CD28 in muizen te verhinderen wordt door veel onderzoekers CTLA4-Ig gebruikt. Wanneer muizen CTLA4-Ig (vrij CTLA4) toegediend krijgen, zal dit molecuul aan de B7-1 en B7-2 moleculen binden waardoor deze liganden niet meer aan CD28 kunnen binden. In studies van diverse onderzoekers is aangetoond dat behandeling van muizen met CTLA4-Ig ten tijde van de sensibilisatie en/of provocatie (= toediening tijdens de inductie van astma symptomen), het ontstaan van deze symptomen voorkomt. Bij astmapatiënten is het echter gebruikelijk dat ze pas nà het ontstaan van hun luchtwegklachten met een behandeling beginnen. Daarnaast staan veel patiënten onafgebroken bloot aan de stof waar ze allergisch voor zijn. De resultaten van studies waarbij CTLA4-Ig werd toegediend tijdens de inductie van astma symptomen hoeven dus niet representatief te zijn voor de klinische situatie.

In de studie beschreven in hoofdstuk 3 is onderzocht of behandeling met CTLA4-Ig *bestaande* astma symptomen kan verminderen. Hiervoor werd CTLA4-Ig pas nà inductie van de astma symptomen aan de muizen toegediend terwijl de blootstelling aan ovalbumine provocaties werd voortgezet (het zogenaamde “chronische” model).

In muizen behandeld met CTLA4-Ig was het aantal eosinofielen in de longen en de hoeveelheid ovalbumine-specifiek IgE in het bloed met respectievelijk 83% en 43% geremd. Toediening van CTLA4-Ig was effectiever dan de "gouden standaard" corticosteroïde dexamethasone omdat toediening hiervan alleen het aantal eosinofielen in de longen met 97% verminderde en geen effect had op de hoeveelheid ovalbumine-specifiek IgE.

Behandeling met CTLA4-Ig in het "chronische" model was echter minder effectief vergeleken met toediening tijdens de inductie van de astma symptomen. Het is *in vitro* (in de reageerbuis) aangetoond dat T-cellen bij een tweede stimulatie minder co-stimulatie via CD28 nodig hebben dan naïeve T-cellen. De verminderde effectiviteit van CTLA4-Ig in het "chronische" model kan wellicht verklaard worden doordat niet alleen naïeve-, maar ook veel reeds geactiveerde ovalbumine-specifieke T-cellen aanwezig zijn op het moment van CTLA4-Ig toediening. Deze reeds geactiveerde T-cellen kunnen waarschijnlijk ook via een van de vele andere co-stimulatie moleculen geactiveerd worden (zie ook hoofdstuk 4).

Astmapatiënten verschillen onderling veel van elkaar, zowel genetisch als in de ernst van hun ziekte. Daarom is het gebruik van diverse muizenstammen en protocollen voor inductie van de astma symptomen erg zinvol. In bovengenoemde studie is CTLA4-Ig ook toegediend in een "chronisch" model met "ernstiger" kenmerken van allergisch astma. Voor dit model werd een ander protocol gebruikt waardoor er twee keer zoveel eosinofielen in de longen en ongeveer tien keer zoveel ovalbumine-specifiek IgE in het bloed terechtkomen vergeleken met het tot nu toe beschreven "milde" model. In dit "chronische" model voor allergisch astma bleek behandeling met CTLA4-Ig minder effectief te zijn; alleen het aantal eosinofielen in de longen was verminderd met 40%, terwijl de hoeveelheid ovalbumine-specifiek IgE onveranderd bleef. Dexamethasone behandeling van de muizen remde het aantal eosinofielen in de longen met 73%.

Hoofdstuk 4

Korte tijd nadat wij onze experimenten met CTLA4-Ig in de "chronische" modellen voor allergisch astma hadden uitgevoerd, werd het aan CD28 verwante T-cel co-stimulatie molecuul ICOS ontdekt. Op basis van de talrijke eerste studies naar de rol van ICOS bij de T-cel activatie, werd al snel duidelijk dat dit molecuul een mogelijke co-stimulator van reeds geactiveerde T-cellen zou kunnen zijn. In tegenstelling tot CD28, komt ICOS namelijk alleen voor op het oppervlak van geactiveerde T-cellen. Uit studies naar de rol van ICOS in muizenmodellen voor allergisch astma, bleek dat ICOS een grotere rol speelt aan het einde van de provocatie periode (wanneer veel allergeen-specifieke T-cellen geactiveerd zijn) dan tijdens de sensibilisatie (wanneer de T-cellen nog naief zijn).

In de studie beschreven in hoofdstuk 4 zijn de effecten van ICOS blokkade tijdens de inductie van astma symptomen vergeleken met de effecten van ICOS blokkade nà de inductie periode. Blokkade van ICOS tijdens de inductie leidde tot een vermindering in het aantal ontstekingscellen in de longen, maar niet tot een verandering in de hoeveelheid ovalbumine-specifiek IgE in het bloed en de luchtweghyperreactiviteit voor methacholine. In het "chronische" model had blokkade van ICOS echter geen enkel effect op de astma symptomen. In onze modellen voor allergisch astma lijkt ICOS dus geen rol te spelen bij de activatie van reeds geactiveerde ovalbumine-specifieke T-cellen. Deze resultaten lijken in tegenspraak te zijn met eerdere studies naar de rol van ICOS in muizenmodellen voor allergisch astma. Mogelijk liggen verschillen in de uitvoering van de experimenten (gebruikte muizenstam, protocol voor inductie van de astma symptomen) ten grondslag aan de tegengestelde uitkomsten. Aanvullende experimenten zijn noodzakelijk om de rol van ICOS in allergisch astma verder op te helderen.

Hoofdstuk 5

In vitro is aangetoond dat een naïve T-cel die alleen een signaal via de T-cel receptor ontvangt zonder co-stimulatie, de eerst volgende keer wanneer hij wel beide signalen krijgt, niet kan reageren. Het blokkeren van co-stimulatie moleculen bij orgaan transplantatie in proefdieren heeft in sommige studies geleid tot langdurige acceptatie van het transplantaat (tolerantie). Dit houdt in dat de T-cellen ook geremd waren nadat de stoffen die de co-stimulatie moleculen blokkeerden uit het lichaam verdwenen waren. Deze resultaten zouden kunnen betekenen dat ook bij astma een kortdurende behandeling met co-stimulatie blokkade leidt tot tolerantie voor het allergeen en dus langdurige verbetering van de symptomen.

In de studie beschreven in hoofdstuk 5, is onderzocht of blokkade van T-cel co-stimulatie moleculen tijdens de inductie van de astma symptomen tot langdurige verbetering van deze symptomen leidt. Dat wil zeggen, niet alleen een verbetering van de astma symptomen direct na de inductie maar ook elf weken later nadat de muizen opnieuw aan ovalbumine provocaties worden blootgesteld (de tweede provocatie periode) als de blokkerende stoffen uit het bloed van de muizen verdwenen zijn. In sommige andere studies werd aangetoond dat voor het ontstaan van tolerantie het blokkeren van CD28 in combinatie met blokkade van CD40 Ligand (een ander bekend T-cel co-stimulatie molecuul) effectiever is dan het blokkeren van één van beide moleculen afzonderlijk. Daarom zijn in de studie beschreven in hoofdstuk 5, CTLA4-Ig en een antistof tegen CD40 Ligand zowel afzonderlijk als in combinatie toegediend.

In overeenstemming met eerdere studies, voorkomt behandeling van muizen met CTLA4-Ig tijdens de inductie van astma symptomen het ontstaan hiervan. Na

de tweede provocatie periode, elf weken later, was het aantal eosinofielen in de longen nog steeds verminderd. In het “milde” muizenmodel voor allergisch astma was na elf weken niet alleen het aantal eosinofielen in de longen maar ook de hoeveelheid ovalbumine-specifiek IgE in het bloed afgenomen. In beide modellen was er geen lange-termijn effect op de luchtweghyperreactiviteit voor methacholine. Kennelijk zijn niet alle astma symptomen even gevoelig voor het ontstaan van tolerantie.

Toediening van de antistof tegen CD40 Ligand bleek na de inductie periode alleen de toename van ovalbumine-specifiek IgE in het bloed te voorkomen en had geen enkel lange-termijn effect. Behandeling met de combinatie was net zo effectief als behandeling met alleen CTLA4-Ig.

Conclusies

Muizenmodellen voor allergisch astma hebben al veel bijgedragen aan de huidige kennis van de onderliggende mechanismen die aan het ontstaan van deze ziekte bijdragen. Experimentele therapieën die in de muis zeer succesvol waren, bleken bij astmapatiënten helaas soms minder succesvol te zijn. Dit kan veroorzaakt zijn doordat het gebruikte muizenmodel te weinig overeenkwam met de klinische situatie. Om de voorspelbaarheid van onze studies te vergroten is daarom het “chronische” model ontworpen en zijn sommige experimenten zowel in een “mild”- als in een “ernstiger” model voor allergisch astma uitgevoerd.

In alle studies was blokkade van CD28 door CTLA4-Ig in muizenmodellen voor allergisch astma het meest effectief vergeleken met blokkade van ICOS en CD40 Ligand. CTLA4-Ig lijkt dus de beste therapeutische mogelijkheden te hebben. Vanwege de belangrijke rol van CD28 bij het ontstaan van astma symptomen in de muis (hoofdstuk 2) zou CTLA4-Ig een alternatieve therapie kunnen zijn voor astmapatiënten die enige tijd niet aan het allergeen hebben blootgestaan zoals bij seizoensgebonden allergieën. Het aantal T-cellen die geactiveerd zijn geweest moet dan wel behoorlijk zijn afgenomen in die periode zodat het aantal (nieuwe) naïeve cellen die voor hun stimulatie sterk afhankelijk zijn van co-stimulatie groter is. Tijdelijke blokkade van CD28 zou bij deze patiënten zelfs tot langdurige verbetering van sommige astma symptomen kunnen leiden (hoofdstuk 5). In de mens is echter nog weinig bekend over de levensduur van geactiveerde T-cellen en de bijdrage van nieuwe naïeve allergeen-specifieke T-cellen aan de ziekte.

Voor astmapatiënten met symptomen, zal behandeling met alleen CTLA4-Ig waarschijnlijk onvoldoende zijn. Het aantal eosinofielen in de longen en de hoeveelheid ovalbumine-specifiek IgE in het bloed was slechts gedeeltelijk geremd door behandeling met CTLA4-Ig in het “chronische” muizenmodel voor allergisch astma (hoofdstuk 3). Vooral mensen met ernstiger vormen van astma zullen extra

medicatie nodig hebben naast CTLA4-Ig omdat een duidelijk verschil in effectiviteit van CTLA4-Ig tussen het “milde” en “ernstiger” muizenmodel voor allergisch astma werd gevonden. Omdat de resultaten van CTLA4-Ig toediening in het “chronische” model even goed of zelfs beter waren dan dexamethasone toediening, zou CTLA4-Ig echter een alternatief kunnen zijn voor corticosteroïden die bij een hoge dosis veel bijwerkingen geven. Dit geldt ook voor astmapatiënten die ongevoelig zijn voor corticosteroïden.

DANKWOORD / ACKNOWLEDGEMENTS

Lieve God, dank U wel voor alle mogelijkheden die ik heb gekregen en mensen die ik heb ontmoet:

Antoon, ten eerste was het erg prettig dat je een goed AiO-project hebt geschreven waarvan je de voortgang op gepaste afstand in de gaten hield. Daarnaast was het leuk om met jou discussies over onze resultaten te voeren. Ik bewonder je snelle en creatieve manier van denken. Tijdens het schrijven vond ik je aanwijzingen erg stimulerend.

Betty, jouw bereidwillige en flexibele instelling en vrolijke gezelschap waren ideaal voor de grote *in vivo* experimenten. Daarnaast heel veel dank voor alle ELISA's, getelde coupes, challenges in de weekenden enzovoort...

Gerard, zeer bedankt voor alle hulp als "stand-in" en je bijzondere diensten zoals het i.v. spuiten en vertalingen naar het Frans.

Frans, jij hebt de basis gelegd voor een groot en goed geoutilleerd lab waar iedereen welkom is. Knap zoals jij tijdens mijn werkbesprekingen snel waardevol advies wist te geven.

Het was erg leuk om op een groot lab te werken zodat ik veel verschillende mensen heb leren kennen. Ik wil iedereen bedanken voor alle hulp en bovenal de dagelijkse gezelligheid en soms zelfs vriendschap.

Mariëtte, Miranda en Frans, bedankt voor de muizen, hulp en discussies. Heel leerzaam om experimenten aan co-stimulatie op een ander lab van dichtbij mee te maken.

Linda Burkly, Anthony Coyle and Paula Jardieu, thank you for providing essential materials for our experiments and the helpful discussions.

Ouders, (schoon)familie en vrienden ("En, hoe gaat het met de muizen?"), heerlijk dat jullie er zijn.

Joost, heb ik vandaag al gezegd dat jij de liefste bent?

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 18 januari 1974 te Zwolle. In juni 1992 behaalde zij het gymnasium β diploma aan het Gymnasium Celeanum te Zwolle.

In datzelfde begon zij met de studie Farmacie (tegenwoordig Farmaceutische Wetenschappen) aan de Universiteit Utrecht waarvan zij in december 1997 het doctoraalexamen behaalde. Gedurende de doctoraalfase werden twee onderzoeksstages voltooid. De eerste onderzoeksstage werd uitgevoerd op het Laboratory for Molecular Pharmacology van het Rigshospitalet in Kopenhagen, Denemarken onder begeleiding van Prof. dr. Thue W. Schwartz. Daar deed zij gedurende zeven maanden onderzoek naar de signaal-transductie paden gekoppeld aan de neurokinine-1 receptor. Een tweede stage van zes maanden werd uitgevoerd op het Laboratory for Developmental Neurobiology van de National Institutes of Health in Bethesda, Maryland, Verenigde Staten onder begeleiding van Dr. Steven L. Coon en Dr. David. C. Klein. Zij werkte mee aan de klonering van het gen voor een serotonine N-acetyltransferase enzym in de snoek (*Esox lucius*) en bepaalde vervolgens de kinetische eigenschappen van dit enzym.

Vervolgens was zij vanaf maart 1998 werkzaam als assistent-in-opleiding bij de disciplinegroep Farmacologie en Pathofysiologie van de Faculteit Farmaceutische Wetenschappen van de Universiteit Utrecht. Daar werkte zij in samenwerking met ing. Betty C.A.M Lobato-van Esch onder begeleiding van Dr. Antoon J.M. van Oosterhout en Prof. dr. Frans P. Nijkamp aan een onderzoeksproject gefinancierd door het Nederlands Astma Fonds. De resultaten van dit onderzoek staan beschreven in dit proefschrift.

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