CD28 and IL-4: two heavyweights controlling the balance between immunity and inflammation

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Abstract The costimulatory receptor CD28 and IL-4Rα-containing cytokine receptors play key roles in controlling the size and quality of pathogen-specific immune responses. Thus, CD28-mediated costimulation is needed for effective primary T-cell expansion and for the generation and activation of regulatory T-cells (Treg cells), which protect from immunopathology. Similarly, IL-4Rα signals are required for alternative activation of macrophages, which counteract inflammation by type 1 responses. Furthermore, immune modulation by CD28 and IL-4 is interconnected through the promotion of IL-4 producing T-helper 2 cells by CD28 signals. Using conditionally IL-4Rα and CD28 deleting mice, as well as monoclonal antibodies, which block or stimulate CD28, or mAb that deplete Treg cells, we have studied the roles of CD28 and IL-4Rα in experimental mouse models of virus (influenza), intracellular bacteria (L. monocytogenes, M. tuberculosis), and parasite infections (T. congolense, L. major). We observed that in some, but not all settings, Treg cells and type 2 immune deviation, including activation of alternative macrophages can be manipulated to protect the host either from infection or from immunopathology with an overall beneficial outcome. Furthermore, we provide direct evidence that secondary CD8 T-cell responses to i.c. bacteria are dependent on CD28-mediated costimulation.

Keywords CD28 · Costimulation · IL-4 · IL-4R · Alternatively activated macrophages · Mouse models · Conditional knockout · Monoclonal antibodies · Regulatory T-cells · Influenza · L. monocytogenes · M. tuberculosis · T. congolense · L. major
Introduction

An immune response to an infection can be called successful (a) if the invading pathogen is eliminated or at least effectively controlled and (b) if this aim is reached without excessive damage to the host. To insure success, tight qualitative and quantitative regulation of the immune response is needed. Thus, different types of pathogens call for different arms from the warehouse of immune effector mechanisms. Overshooting responses are avoided by an equally diverse set of counter-regulating measures. The choice of the appropriate weapon against an invader becomes manifested in terms of T-helper subset differentiation. However, this is guided by signals from innate immune cells generated in response to pathogen class recognition via PAMPs. Limitation of immune responses is achieved by antigen and growth factor deprivation (with the exception of chronic infections), the induction and activity of negative regulators such as CTLA-4, BTLA, or PD1 in cis, i.e., on the effector cells themselves, and by “regulatory” T-cells (Treg cells) and “alternatively activated” macrophages (aaMphs) acting as dominant suppressor cells in trans.

Among the cytokines and costimulatory receptors regulating the balance between a highly effective immune response with the smallest possible collateral damage, the Th2 signature cytokine IL-4 and the costimulator CD28 occupy key positions. This is illustrated by the phenotype of the corresponding knockout mice that show imbalanced responses when challenged with pathogens.

IL-4 and IL-13 share a common signaling pathway through the IL-4 receptor α (IL-4Rα) chain. A functional IL-4R (type 1) requires assembly of IL-4Rα with a γc chain, while interaction of IL-4Rα with an IL-13Rα1 subunit leads to formation of a functional IL-13 receptor (type 2) [1]. Therefore, by blocking or deleting IL-4Rα, IL-4 and IL-13 functions are abrogated. While this is the case for most responses, more recent studies showed some evidence that IL-13 bound to IL-13Rα2, which was believed to be a decoy receptor for IL-13 [2] but may also induce signaling in certain cells under particular conditions [3].

More than a decade ago, constitutive IL-4Rα deficient mice were generated by using homologous recombination in embryonic stem cells [4]. Subsequent studies in this mutant mouse strain, including comparative studies in IL-4 and IL-13 deficient mice, uncovered important biological roles for IL-4Rα-responses mediated by IL-4 and IL-13. It turned out that II-13, believed to be the small brother of IL-13, had overlapping roles, but also strikingly distinct roles, particularly in activating certain effector functions [5].

In the case of CD28, originally defined as the main costimulator of primary T-cell responses, genetic deficiency leads to an impairment of antibody responses (but less of cell-mediated immunity) [6], along with defective Th2 polarization and a marked reduction in regulatory T-cells [7], indicating that CD28-mediated costimulation is of particular importance for Treg and type 2 responses (including alternative macrophage activation via IL-4), while being less critical for cell-mediated immunity.

Importantly, such observations made in constitutively gene-deleted mice are unlikely to reveal the full importance of the relevant molecules in immune regulation due to the enormous plasticity of the immune system during and also after its maturation, which allows adaptational and back-up mechanisms to overcome at least some of the deleterious effects of genetic defects. Furthermore, the role these molecules play when expressed by particular cell types remains unaddressed. Accordingly, interference with the expression or function of immune regulators at the time of infection or in a cell type specific manner has become the most important tool in the analysis of their protective and pathological effects in vivo. Experimentally, this is achieved by “conditional” gene targeting using the Cre/loxP system. This strategy has been successfully employed for the IL-4Rα. Specifically, mice which are selectively IL-4Rα deficient in macrophages and neutrophils [8], smooth muscle cells [9], the CD4+ T-cell subpopulation [10] or T-cells [11] as well as B cells and dendritic cells (unpublished) have been used to probe IL-4Rα function in these cell types after various immune and non-immune challenges. More recently, conditionally deleting CD28 knockout mice have also been generated (F.L. and T.H., unpublished). In addition to this genetic approach, both blocking and stimulatory CD28-specific mAb were developed [12] which allow acute interference with [13] or activation of [14] this receptor during an immune response.

Interference with CD28-mediated costimulation

Conditional CD28 knockout mice were generated by flanking exons 2 and 3 with LoxP sequences, allowing effective deletion when crossed to the appropriate Cre transgenic lines (F.L., unpublished). Importantly, these mice also delete CD28 in an inducible fashion when a tamoxifen-regulated transgene is introduced. For the first time, this will allow to study the importance of CD28 in the recall of memory responses, an area of controversy for many years [15, 16]. Previous attempts to address this issue by monoclonal antibody blockade were hampered by the facts that a) no truly ligand-binding site-specific mAb to CD28 was available (but see below), and b) that blocking of CD80 and CD86, the ligands of CD28, confounded results by also depriving the negative regulator CTLA-4 (CD152) of these same ligands [17]. Similarly, transfer of memory T-cells into CD80/CD86 deficient hosts [17] does...
not allow to attribute the observed effects upon restimula-
tion with the pathogen to a lack of CD28-mediated
costimulation because again, CTLA-4 engagement is also
affected.

First results using *Listeria monocytogenes* infection
indicate that indeed, targeted deletion of CD28 between the
establishment and recall of memory strongly impairs the
secondary CD8 T-cell response to a model antigen
expressed by the Listeria (M.F., T.G., F.L., T.H., unpub-
lished). This is most likely due to a direct effect on the CD8
T-cells because the elimination of CD4 T-cells (including
Treg cells) was shown to result in an increase rather than a
decrease of the secondary CD8 T-cell response in the same
system \[ 18 \]. An example of the results thus obtained is
given in Fig. 1a.

In order to study the importance of CD28-mediated
costimulation for secondary T-cell responses, we have not
only established inducible gene targeting but also the first
mAb to mouse CD28 which fully blocks binding of natural
ligands \[ 12, 13 \]. This mAb was generated in mice, per-
mitting prolonged treatment without the induction of neu-
tralizing antibodies. Indeed, application of this mAb, called
E18, in GvHD \[13\] and in a mouse asthma model (T.G. and
T.H, unpublished) has shown high therapeutic
efficacy in a wide range of rodent models of autoimmunity,
inflammation, and allograft rejection \[25–31\]. Moreover,
CD28SA application expands the T-cell compartment in
lymphopenic hosts, promoting recovery from immunoin-
competence \[32\]. While the disastrous outcome of a first-
in-man-study of a human CD28SA \[33\] has interrupted
further development of CD28SA-based therapeutics,
these antibodies continue to be useful tools for the
manipulation of the rodent immune system in attempts to
study the contribution of immune deviation and/or Treg
activation in the control of immunopathology.

With regard to the effects of CD28SA on the course of
infections and microbe-induced inflammatory responses in
rodent models, the following results have been obtained:

1. **Adjuvant arthritis.** In this model, a Th1 response of LEW
rats to heat-killed mycobacteria in adjuvant results in
swelling and inflammation of joints along with exten-
sive cartilage destruction. Application of the mouse anti-
rat CD28SA JJ316 is highly efficacious in preventing
and even reversing pathology in both preventive and
therapeutic settings \[34\] and unpublished).

2. **Mouse Influenza.** Intranasal infection of mice with the
Influenza A HKx31 (H3N2) leads to transient
weight loss, lung pathology, and high levels of pro-

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**Immune deviation and Treg activation with stimulatory
CD28-specific antibodies**

In the course of generating the first rat CD28-specific mAb,
we discovered a novel class of such mAb which is able to
trigger T-cell activation without the engagement of the
TCR \[19\]. In contrast to conventional CD28-specific mAb,
which bind monovalently close to or at the natural ligand-
binding site, such “CD28 superagonists” (CD28SA) bind
laterally, allowing lattice formation by crosslinking of
individual CD28 homodimers \[20, 21\]. This correlation
between function and topology of binding was observed in
rats, mice, and humans \[12, 20\]. In rodents, CD28 super-
agonists induce immune deviation to Th2 \[22\] as well as
expansion and functional activation of “natural” Treg cells
\[23–25\]. Accordingly, such mAb have high therapeutic
efficacy in a wide range of rodent models of autoimmunity,
inflammation, and allograft rejection \[25–31\]. Moreover,
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2. **Mouse Influenza.** Intranasal infection of mice with the
Influenza A HKx31 (H3N2) leads to transient
weight loss, lung pathology, and high levels of pro-
inflammatory cytokines in the bronchoalveolar lavage and plasma. Application of the mouse anti-mouse CD28SA D665 alleviates weight loss and inflammation without compromising the control of the infection, an effect mapped to Treg activation by its recapitulation when Treg cells from D665-stimulated cells were transferred (Fig. 2 and unpublished observations). Shown are the marked increases in Treg cells in the bronchoalveolar lavage of flu-infected mice under CD28SA treatment (Fig. 2a), as well as the decrease in TNF and the increase in IL-10 in the lung after D665 stimulation or transfer of Treg cells from D665-treated uninfected mice i.v. on day one of infection. BAL was analyzed by ELISA for TNF and IL-10 on day 6.

3. Mouse trypanosomiasis. A similar protective effect against immunopathology via experimental Treg stimulation was observed in mice infected with Trypanosoma congolense [35]. In this model of African trypanosomiasis, CD28SA treatment down-regulated IFNγ and TNF production by T-cells along with the production of reactive oxygen species by classically activated macrophages. Interestingly, it also triggered the development of alternatively activated macrophages, in line with a CD28SA-induced Th2 shift and IL-4 production [22]. With regard to pathology, CD28SA treatment delayed the onset of liver injury, diminished the anemia burden, and prolonged the survival of infected animals.

4. Mouse TB. Here, the TH1 response to inhaled Mycobacterium tuberculosis is required for pathogen containment. CD28SA treatment during M. tuberculosis infection increased the percentage of Treg cells systemically as well as in the lung (Fig. 3b), where it also increased IL-10 production (Fig. 3b) but had no effect on bacterial burden in the lung, liver, or spleen (Fig 3c).

5. Mouse leishmaniasis. As is the case in mouse TB for the intracellular bacterium M. tuberculosis, the control of the parasite burden in Leishmania major depends on a pro-inflammatory Th1 response [36]. We either depleted Treg cells with CD25-specific mAb, or polyclonally stimulated the Treg and Th2 compartment with the CD28SA D665 from week 4 to week 10 post-infection. In both C57BL/6 “healer” and BALB/c “progressor” strains, increased DTH (Th1) responses to Leishmania antigens were observed when Treg cells were depleted after infection, whereas CD28SA treatment decreased the reaction, in line with the role of Treg cells and a type 2 response in this system (Fig. 4a). Furthermore, swelling of the infected footpad (Fig. 4b), parasite load in the infected footpad (Fig. 4c), and antigen-specific IFN-γ production in C57BL/6 mice (Fig. 4d) were negatively influenced by CD28SA treatment and improved by Treg depletion. Progression of L. major infection, DTH responses, and parasite loads in IL-4Rα-/- mice were largely unaffected by CD28SA treatment versus controls (not shown), suggesting that enhancement of type 2 (as compared to Treg) responses may be responsible for the CD28SA effect in C57BL/6 and BALB/c mice (Table 1).
Interference with IL-4Rα-mediated cell signaling

The role of alternatively activated macrophages in infectious and inflammatory disease

Macrophage activation can be divided into a classical and an alternative pathway. IFN-γ-induced, classically activated macrophages (caMphs) are indispensable for protective effector responses against intracellular pathogens, particularly due to their highly effective killing effector function. However, excessive inflammatory immune responses mediated by caMphs can also be detrimental to
the host. In contrast, IL-4Rα-mediated alternative macrophage activation has been proposed as a possible mechanism to attenuate excessive inflammation. The identification of marker genes [37–42] as well as the generation of macrophage/neutrophil-specific IL-4Rα-deficient mice (LysM cre IL-4Rα-/-lox) and subsequent studies of relevant experimental disease models have enabled us to evaluate the role of IL-4Rα-mediated alternative macrophage activation in vivo. Together, these investigations demonstrated the involvement of aaMphs in immunomodulation and suppression during infectious diseases, like schistosomiasis [8], leishmaniasis [43], cryptococcosis [44–46], as well as during non-infectious inflammatory diseases, like proteoglycan-induced arthritis [46, 47], or experimental autoimmune encephalomyelitis (EAE) [47]. Here, they seem to influence both innate and adaptive immune responses, the latter by suppression of T-cell proliferation [48, 49].

The role of IL-4Rα-responsiveness in helminthic diseases

In helminth infection, like *Nippostrongylus* and *Schistosoma*, IL-13 turned out to be the essential factor for efficient expulsion and induction of goblet hyperplasia [8, 50], as well as smooth cell hypercontraction [9]. Further studies in experimental schistosomiasis uncovered, however, that IL-4/IL-13-independent hyperplasia is possible [51], and defined the protective role of alternatively activated macrophages by down-regulation of otherwise overshooting T-cell responses [8]. In addition, the use of mice deficient for IL-4Rα on particular T-cell subsets revealed that both CD4+ Th cells and non-CD4+ T-cells are involved in avoiding early mortality and morbidity during schistosomiasis [10, 11].

Beneficial and detrimental IL-4Rα-responsive cell types in leishmaniasis

Infection studies in CD4+ T-cell-specific IL-4Rα-deficient BALB/c mice with *Leishmania major*, which causes cutaneous leishmaniasis, confirmed the detrimental role of IL-4-promoted Th 2 cells, but also suggested that IL-4Rα-responsive cells are involved in protection against cutaneous leishmaniasis [52]. AaMph’s could be excluded as they rather contribute to susceptibility in BALB/c mice by influencing T-helper responses and facilitating evasion by the parasite [43]. Currently, recently established dendritic cell-specific IL-4Rα-deficient mice are employed to test the possibility that DC instruction via IL-4 and/or IL-13 may be the missing link.

### Table 1 Constitutive and conditional IL-4Rα-deficient mouse models

| Mutant mouse strains | Cell specificity | Status |
|----------------------|------------------|--------|
| **Constitutive mutation** | | |
| IL-4Ra-/- | All cells | Mohrs et al. [4] |
| IL-4Ralox/lox | All cells (silent mutation) | Herbert et al. [8] |
| **Cell type deficiency** | | |
| LysMcreIL-4Rα-/-lox | Macrophages and neutrophils | Herbert et al. [8] |
| SMMHCcreIL-4Rα-/-lox | Smooth muscle cells | Horsnell et al. [9] |
| LckcreIL-4Rα-/-lox | CD4+ T lymphocytes | Leeto et al. [10] |
| iLckcreIL-4Rα-/-lox | All T lymphocytes | Dewals et al. [11] |
| CD4creIL-4Rα-/-lox | a/b+ TCR T lymphocytes | Unpublished |
| MB1creIL-4Rα-/-lox | B lymphocytes | Unpublished |
| CD11ccreIL-4Rα-/-lox | Dendritic cells | Unpublished |
| LysMcreLckcreIL-4Rα-/-lox | Macrophages/neutrophils/CD4+ T cells | Unpublished |
| **Inducible & cell type deficiency** | | |
| TetOIL4RaTg | All cells | Unpublished |
| tTA-VAYTetOIL4RaTgIL-4Rα-/- | Haematopoetic cells | Unpublished |

Concluding remarks

CD28 and the IL-4Rα highlight the potency of costimulatory receptors and cytokines in fine-tuning immune responses to pathogens while avoiding host damage. While “knockout” and blocking experiments defined important roles for CD28 in T-cell memory and for IL-4R signaling in the activation of an anti-inflammatory type of macrophages, the application of stimulatory CD28-specific mAb makes use of two major host-protective pathways by boosting Treg and Th2 activation, which in turn leads to alternative activation of macrophages. Notably, in several of the infectious disease models we have studied, it was
possible to use such mAb treatment to protect the host from immunopathology without compromising pathogen control. This is of potential translational interest because we found that high-dose corticosteroid treatment does not interfere with the activation of Treg cells by CD28SA in vivo [14], opening the possibility of controlling a potential cytokine storm while maintaining the beneficial activation of this host-protective T-cell subset.

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