ICOS and CD28: similar or separate costimulators of T cells?

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ICOS en CD28: vergelijkbare of verschillende costimulators van T cellen?
(met een samenvatting in het Nederlands)

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You have brains in your head.
You have feet in your shoes.
You can steer yourself
any direction you choose.
— Dr Seuss

Aan ons pap & ons mam

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Chapter 1

CD28 and ICOS: similar or separate costimulators of T cells?

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Summary

Numerous studies have revealed that the B7.1/B7.2 - CD28 and B7RP-1 - ICOS (Inducible COStimulator) pathways provide crucial costimulatory signals to T cells. We have compared the contribution of these pathways during primary and effector responses, *in vitro* and *in vivo*, molecularly as well as functionally. This comparison between CD28 an ICOS after initiation of T cell activation demonstrates that both CD28 and ICOS function similarly during expansion, survival and differentiation of T cells and that both CD28 and ICOS are necessary for proper IgG responses. The major differences between CD28 and ICOS are differences in expression of both receptors and ligands, and the fact that CD28 induces IL-2 production, whereas ICOS does not. In addition, ICOS is more potent in the induction of IL-10 production, a cytokine important for suppressive function of T regulatory cells. All data available at present indicate that both molecules are very suitable candidates for immunotherapy, each in their own unique way.

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Introduction

Antigen (Ag)-specific recognition by the TCR receptor is not sufficient to activate T cells. In addition, signals provided by costimulatory molecules are mandatory for optimal T cell activation. An appropriate T cell response requires optimal balance between activatory and inhibitory signals. Over the last decade, a number of molecules belonging to the B7-family of costimulators has been discovered that can conduct either positive or negative signals in addition to the Ag-specific signal into T cells (reviewed in Ref. 1). The best characterized ligand-receptor pairs are B7.1/B7.2 (CD80/CD86)-CD28/CTLA-4 (CD152). Being expressed on naïve T cells, CD28 is the only activating receptor of the B7-family for these T cells. $CD28^{-/-}$ mice revealed pronounced immune defects, clearly evident in diminished T cell dependent humoral responses (2). CTLA-4 (cytotoxic T lymphocyte antigen-4) is rapidly upregulated after T cell activation and serves as an attenuator for T cell responses (3). Both receptors bind to ligands B7.1 and B7.2 expressed on the antigen presenting cell (APC), albeit with different affinities and with different kinetics. Another important costimulation ligand-receptor pair is B7RP-1 (ICOSL, B7h, B7-H2, GL50)-ICOS (Inducible COstimulator). The phenotype of $ICOS^{-/-}$ mice showed striking resemblance to that of $CD28^{-/-}$ mice, suggesting an important costimulatory function for T helper effector functions and T:B cell help (4-6).

Other members of the B7 costimulatory family of molecules include the negative regulators PD-1, with its ligands PD-L1 (B7-H1) and PD-L2 (B7-DC), and the recently discovered CD28 homologue BLTA with a yet to be discovered ligand (7-10). In addition, two novel B7 homologues B7-H3 (B7RP-2) and B7-H4 (B7x, B7S1), both binding to an unknown receptor on T cells have been described to attenuate T cell responses (11-14). Next to members of the B7-costimulatory family, other surface molecules have also been found to provide costimulatory signals in addition to the TCR, such as TNF-related family members (CD27, 4-1BB, CD40L), adhesion molecules (LFA-1, CD2) or CD4 (15-17). The eventual activation state of the T cell is dependent on the accumulation and net result of all activatory and inhibitory signals. With an abundance of both positive and negative costimulators of T cells it is of great interest to understand the contribution of each ligand-receptor pair to a T cell response and whether they have distinct or overlapping functions. In this review we focus on comparing two activatory receptors of the B7-family, CD28 and ICOS.

Within the B7-family, CD28 and ICOS are most homologous with respect to structure and function. Both are type I transmembrane receptors expressed as homodimers, with an extracellular (Ig)V-like domain, a hallmark of receptors of the B7 related family. CD28 and ICOS share 19% (murine) and 24% (human) amino acid (AA) homology (18, 19), which is common among all functionally related members of this family. Moreover, the ICOS and CD28 genes are located on the same chromosome adjacent to each other, which suggests that gene duplication has occurred in evolution (20-22). Next to the clear immune defects observed in the single knockout mice, recent studies in CD28-ICOS double knockout mice demonstrated even more

profound compromised T cell responses.

Mice defective for both pathways showed severely impaired humoral responses against virus, protein antigens (Ags) and environmental Ags (23), indicating that both CD28 and ICOS together are mandatory to drive an *in vivo* T cell dependent B cell response. Although the function of ICOS and CD28 on $CD8^+$ T cells is well established, this review focuses on the differences and similarities between CD28 and ICOS with respect to their role in a $CD4^+$ T cell response.

B7.1/2-CD28 and B7RP-1-ICOS expression in space and time

One way to control the function of costimulatory molecules in T cell activation is to tightly regulate their expression. An important difference between CD28 and ICOS is their expression pattern. In both mice and humans, CD28 is constitutively expressed at constant levels on both naïve and activated T cells, as well as differentiated T cells. In humans, only one specific subpopulation of Ag-experienced $CD4^+$ T cells lack expression of CD28 (24, 25). Compared to CD28, ICOS expression is more variable. ICOS expression is low on naïve human and murine cells and is upregulated within hours after TCR engagement (18, 19, 26). After activation, ICOS expression persists on recently activated as well as memory Th1 and Th2 $CD4^+$ T cells (27, 28). In addition to the TCR signal, cytokines such as IL-2 and IL-4 in murine T cells, and IL-12 and IL-23 in human T cells can further enhance ICOS expression (29, 30). Although CD28 coligation is not necessary for induction of ICOS expression, it can promote ICOS levels (31, 32). Of note, ICOS expression appears to be higher on Th2 $CD4^+$ T cells compared to Th1 $CD4^+$ T cells (26, 27). *Ex vivo*, the levels of ICOS expression correlate with a cytokine production pattern; peripheral T cells are either IL-10 producing (high ICOS), IL-4, IL-5 and IL-13 producing (medium ICOS) or IL-2, IL-3, IL-6 and IFN-γ producing cells (low ICOS) (33). Interestingly, this is in concordance with the recent finding that ICOS expression is high on IL-10 producing regulatory T cells (Treg), which require presence of ICOS for optimal regulatory function (34, 35).

The expression of the B7 ligands is tightly and differentially regulated. B7.1 and B7.2 expression is restricted to professional APCs, e.g. immature dendritic cells (DCs), monocytes, macrophages, and B cells. B7.2 is, unlike B7.1, constitutively expressed at low levels on B cells, monocytes and DCs (36-38). Upon activation by different kinds of stimuli both B7.1 and B7.2 are upregulated, although B7.2 expression is more rapidly enhanced compared to B7.1. Like B7.1/2, B7RP-1 (the ligand of ICOS) can be induced on macrophages and monocytes (39, 40). In contrast to B7.1/2, B7RP-1 is highly expressed on immature DCs and B cells and can even be downregulated upon stimulation with bacterial stimuli and BCR signaling or IL-4 respectively (41, 42). Moreover, B7RP-1 expression is not restricted to haematopoietic cells. It can be induced on fibroblasts and epithelial cells after addition of inflammatory stimuli such as LPS, TNFα and IL-1β (43, 44). In conclusion, B7-CD28 and B7RP-1-ICOS receptor pair show distinct expression profiles, some of which may account for the functional differences described below.

CD28 and ICOS intracellular tails compared

TCR triggering provided with appropriate costimulation initiates a series of molecular events in signaling cascades eventually leading to gene transcription in the nucleus. In general, B7-related costimulatory molecules are considered to modify the TCR signal, rather than delivering qualitatively different signals in the cell (45, 46) (reviewed in Ref. 7). Although CD28 has been implicated to initiate unique signaling pathways, TCR and CD28-induced signals converge before or at the level of the nucleus (**Fig. 1**). Dissecting signaling cascades leading to gene expression may elucidate the function of either CD28 or ICOS: do they each amplify unique signals initiated by the TCR or do both molecules amplify similar signals? Looking at binding partners of the intracellular tails may provide clues as to which signaling pathways are downstream of each molecule.

The CD28 intracellular tail consists of 41 amino acids (AA) containing multiple putative binding motifs for signaling molecules (reviewed in Ref. 48). Four tyrosine residues are present in the CD28 tail, which are phosphorylated upon T cell activation. Of these tyrosine residues, one

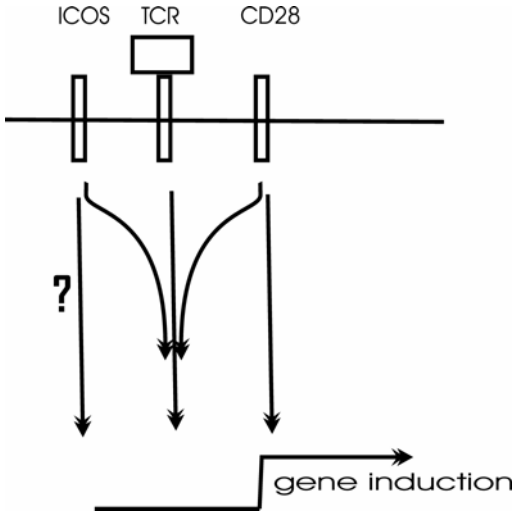

Figure 1. Costimulatory signals converge before or at the level of the nucleus with signals generated by the TCR.

(Tyr170) is embedded into a highly conserved YxxM (Y_{170}MNM) motif, consensus for Src-homology 2 (SH2) containing proteins shared by CTLA-4 and ICOS (**Fig. 2**). Furthermore, the CD28 cytoplasmic tail contains two proline rich regions each of which conforms to the consensus PxxP SH3 binding sequence, which are absent in the ICOS intracellular tail. The p85 subunit of PI3K can associate with phosphorylated Tyr170 within the YMNM motif of the CD28 intracellular tail (49). Tyr170 mediates several functions of CD28, such as proliferation and cell survival, possibly by induction of Bcl-X_L via

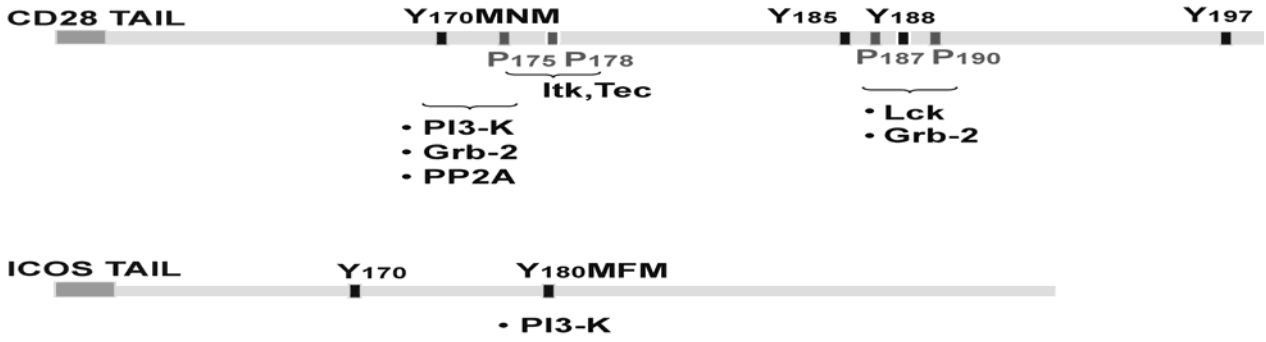

Figure 2. Schematic representation of murine CD28 and ICOS intracellular tails. Shown are tyrosine (Y) and proline (P) residues that serve as docking sites for the proteins depicted.

a PKB dependent pathway, but is dispensable for IL-2 production (50). Other molecules have also been found to associate within the YMNM motif, such as Grb-2 (growth factor receptor bound protein-2) and phosphatase PP2a. In the CD28 tail, Asp172 (N) and the proximal proline rich region are mandatory for SH2- and SH3 domain association of Grb-2 respectively (51). Grb-2 is complexed to Sos (son of sevenless), an activator of RAS, and is involved in IL-2 production (51). The phosphatase PP2a associates with the unphosphorylated Tyr170 and is implicated to negatively regulate CD28 signaling (52). The CD28 COOH terminal PYxPP motif ascertains binding of SRC-family kinase Lck (53), mediating tyrosine phosphorylation of CD28 itself thereby facilitating binding of PI3K, Grb-2 and activation of Itk (IL-2 inducible T cell kinase) (54, 55). Moreover, Itk itself and Tec (Tyrosine kinase expressed in hepatocellular carcinoma) physically associate with the proline motifs in the CD28 intracellular tail, which regulate a variety of downstream events through activation of PLC-γ1 and Erk (56). Both Itk and Tec could be mediating signals leading to T helper cell differentiation, since the PYxPP region proved to be essential for IL-4 and IFN-γ production in primary T cells (57). Interestingly, this proline-rich region in the CD28 intracellular tail appeared to be mandatory for the CD28 induced development of Treg in the thymus (81).

The intracellular tail of ICOS is shorter (21 AA) compared to the CD28 tail and contains two tyrosine residues of which one (Tyr180) lies within the YxxM (Y_{180}MFM) motif. Like CD28, phosphorylation of Tyr180 leads to recruitment of PI3K, although the implication of this interaction remains to be determined (27). The one AA difference at position three between CD28 and ICOS in the common YxxM motif was implicated to explain the lack of IL-2 production upon ICOS signaling, since no Grb-2 could be recruited to this motif in the ICOS tail (58). Recently, we have identified a novel binding partner of both ICOS and CD28, Hax-1, which is important in CD28 induced IL-2 production (manuscript in preparation). The binding to both ICOS and CD28 intracellular tails suggests a common role for Hax-1 in enhancement of the TCR signal, perhaps potentiated further by Hax-1 association to HS-1, a molecule involved in TCR signaling (59). Compared to CD28, ICOS is devoid of the distal region present in the CD28 tail, which encompasses the proline rich regions, three additional tyrosine residues and consequently the associating molecules.

Despite differences in intracellular associating molecules, common signaling pathways are shared by CD28 and ICOS in peripheral T cells. Phosphorylation of Erk1/2, PKB, PDK1 and p38 occurs after CD28 stimulation as well as after ICOS stimulation, possibly through PI3K activation (60). In contrast, JNK (Jun N-terminal kinase) is not activated upon ICOS signaling, which might be consequence of the lack of Grb-2 recruitment. This correlates with the difference between CD28 and ICOS in the induction of IL-2, since JNK activation leads to activation of protein-1 (AP-1) complex, a transcription complex required for IL-2 gene transcription (61).

Genes induced by CD28 and ICOS

What are the consequences of these similar downstream pathways: are similar genes or a distinct array of genes induced by the two different receptors? Using gene profiling studies, we and others have investigated target genes that are differentially regulated by ICOS compared to CD28 in murine CD4+ T cells (**Fig. 3**) or human CD4+ T cells (46). Genes upregulated by ICOS were very similar to genes upregulated by CD28 both in human and in murine stimulated CD4+ T cells. In general, the magnitude of upregulation of genes by ICOS was less

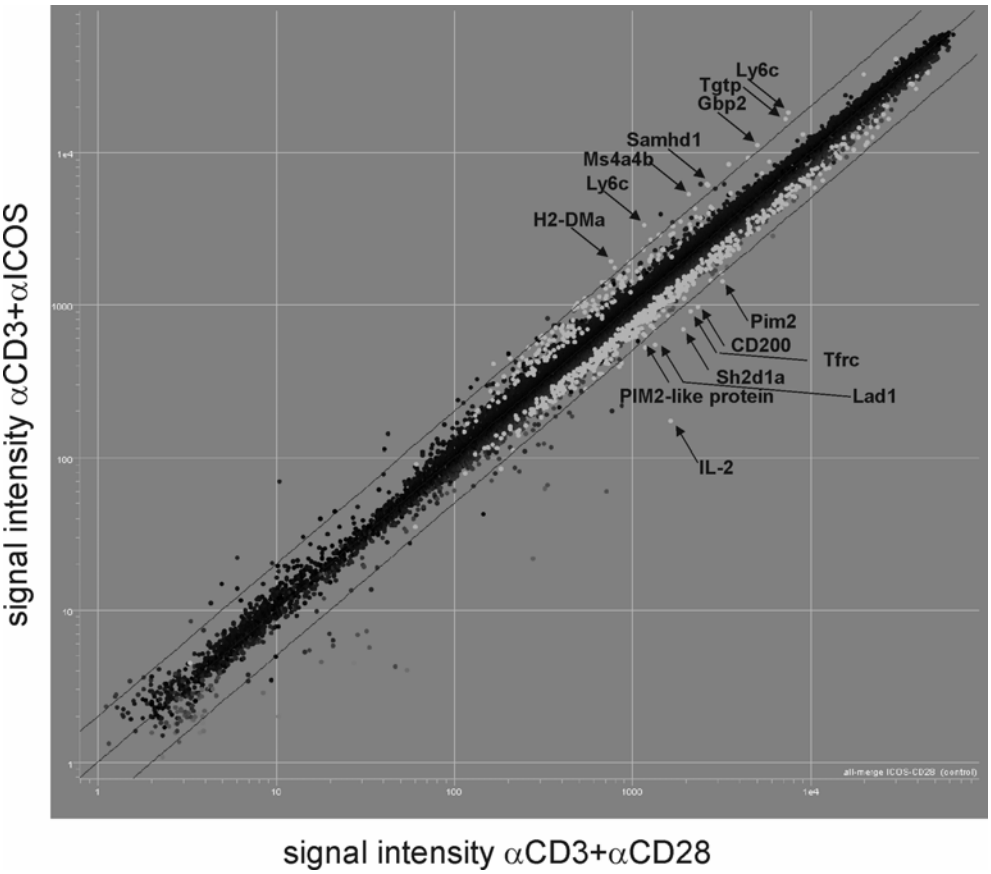

Figure 3. Gene expression profiles after ICOS and CD28 costimulation of CD4+ T cells. Purified murine CD4+ T cells were isolated from lymph nodes and stimulated with *α*CD3-*α*CD28 Ab-coated beads and *α*CD3-*α*ICOS Ab coated beads for 24 hours. For optimal expression of ICOS, IL-2 was added to the cultures. mRNA was amplified and microarray analysis was performed as described by Roepman *et al.* (90). Cy3 and cy5 labeled cRNAs were combined and hybridized on spotted microarrays (Operon, AROS Mouse Version 3). Data shown is average of 4 independent experiments each of which was performed with dye-swap as described (91). Indicated is the diagonal line at which the genes regulated are similar and perpendicular to that 2-fold changed. Grey dots represent the genes significantly changed (analyzed with SAM-oneclass over the 4 averaged dye-swaps (92). Depicted with arrows are the genes reproducibly more than 2-fold upregulated or downregulated by *α*CD3-*α*ICOS compared to *α*CD3-*α*CD28 with a false discovery rate of less than 5%. *H2-Dma*: Class II histocompatibility antigen, m alpha chain precursor; *Samhd1*: SAM domain and HD domain-containing protein 1; *Ly6c*: lymphocyte Ag 6c; *Tgtp*: T-cell specific GTPases; *Gbp2*: Guanylate nucleotide binding protein 2; *Ms4a4b*: Membrane-spanning 4-domain-a-6b; *IL-2*: Interleukin-2; *Pim2*: serine/threonine-protein kinase Pim-2; *Lad-1*: Ladinin-1; *Tfrc*: Transferrin receptor protein-1; *Sh2d1a*: SH2 domain protein-1a.

compared to CD28. In human $CD4^+$ T cells, only IL-2, IL-9 and ICOS itself were upregulated to a greater extend by CD28 compared to ICOS. In murine $CD4^+$ T cells, only six genes were positively regulated in αCD3-αCD28 compared to αCD3-αICOS stimulated cells, among which IL-2 was most pronounced (average of 10-fold increase) (**Fig. 3**). For ICOS costimulated cells, six to eight genes were reproducibly induced at a 2- to 3-fold higher level compared to CD28 stimulated cells. One should bear in mind that the approach of directly comparing αCD3-αICOS with αCD3-αCD28 stimulated cells in gene array analysis does not distinguish between genes that remain similar after αCD3-αCD28 stimulation and upregulated after αCD3-αICOS or genes that are downregulated by αCD3-αCD28 and remain similar in αCD3-αICOS stimulated cells. Nevertheless, these data are in accordance with data published by Riley *et al.*, which indicate that upregulation of many downstream target genes are shared between CD28 and ICOS, and that IL-2 is one gene that is specifically regulated by CD28 (46). In conclusion, other than the marked difference in IL-2 gene induction, the differences in the intracellular tail between ICOS and CD28 seem to result only in differential magnitude of gene expression. Whether similar regulation of genes also results in similar functional consequences of ICOS triggering compared to CD28 ligation will be discussed next.

CD28 and ICOS: *in vitro* T cell response

Subsequent to the gene profiling, we have studied CD28 and ICOS function *in vitro*. CD28 is the major activator of naïve T cells resulting in high proliferative capacity, IL-2 production and induction of cell survival. This is most clearly seen in CD28-deficient T cells, which are refractory to T cell proliferation in response to Ag in the presence of APCs (2, 62, 63). ICOS deficient T cells have no such obvious impairment, although some report diminished proliferative capacity in splenic cultures with anti-CD3 Ab (6, 64). Conversely, murine T cells proliferate when stimulated via TCR and agonistic ICOS signals, but only in the presence of supplemental IL-2 (31). In contrast to murine cells, human peripheral T cells can readily be activated *in vitro* by co-crosslinking the TCR and ICOS simultaneously resulting in T cell proliferation (65). Vice versa, blocking ICOS results in reduced T cell proliferation, even though blocking CD28 is more effective (40).

Next to induction of proliferation, ICOS has been implicated to contribute to T cell survival. Cell death was decreased when T cells were stimulated through the TCR and ICOS simultaneously (65, 66). We have demonstrated that ICOS stimulated cells are equally viable as CD28 stimulated cells, again indicating a role for cell survival. In addition, we have shown that ICOS induces similar expression of the cell survival genes Bcl-X_L and A1, like observed after CD28 ligation, which could be a prerequisite for the induction of cell survival (manuscript in preparation).

On recently activated T cells, where ICOS and CD28 are both highly expressed, ICOS and CD28 induce similar amounts of cytokines, indicative for T cell differentiation. *In vitro* costimulation with either CD28 or ICOS results in cytokine production characteristic for T helper subsets such as IFN-γ, TNFα (Th1) and IL-

4, IL-10, IL-5, IL-13 (Th2) and IL-17 (Th17) (19, 67). Conversely, T cells from $CD28^{-/-}$ and $ICOS^{-/-}$ mice are defective in production of both Th1 and Th2 restricted cytokines, although the IL-4 defect is most pronounced in both. Interestingly, ICOS was found to superinduce IL-10 in human peripheral T cells and IL-10 was also produced by murine T cells expressing high levels of ICOS (19, 33). In addition, T cells from ICOS-deficient patients show a severe reduction in IL-10 production in response to different stimuli, suggesting a crucial role for ICOS in IL-10 production (68).

Taken together, induction of *in vitro* proliferation, cell survival and differentiation are features that are shared by ICOS and CD28. CD28 is crucial for IL-2 production, while ICOS is implicated in IL-10 production. Although this is informative about the function of these molecules in $CD4^+$ T cells, the contribution of each molecule to an *in vivo* response complexed by the interplay between different cell types is still not clear.

CD28 versus ICOS *in vivo* T cell–dependent immune response

A primary T cell dependent response encompasses initiation by TCR-mediated recognition of the foreign Ag presented by the APC, followed by T cell expansion and differentiation. Subsequently, during the effector phase, differentiated $CD4^+$ T helper cells migrate to the germinal centers and provide necessary help to B cells so that IgG production and isotype switching can occur. Both CD28 and ICOS have been extensively studied during the course of such an *in vivo* response. Yet, most studies report the end result of the response, which is an accumulation of all prior events. Therefore, we made an effort to dissect the roles of CD28 and ICOS during different stages of a specific T cell dependent immune response.

As we have described above, both ICOS and CD28 mediate T cell expansion *in vitro*. The role for CD28 during *in vivo* T cell expansion has been well established (1). In addition, several *in vivo* studies support a role for ICOS in T cell expansion. We demonstrated diminished T cell expansion *in vivo* after blocking ICOS in $CTLA\text{-}4^{-/-}$ mice, although this was less effective compared to CD28 blocking agents (31). Similarly, blocking ICOS resulted in reduced T cell numbers in response to bacterial Ags and allo-Ag and clonal expansion was diminished during the initiation phase of an immunization (69-71). In addition, continuous ICOS triggering by B7RP-1 induces $CD4^+$ T cell expansion *in vivo* in terms of activation and T cell numbers (18, 19, 72). Taken together, next to CD28, also ICOS plays a role in T cell expansion *in vivo*, which consequently affects differentiation and effector functions of $CD4^+$ T cells.

Numerous *in vivo* studies have shown that defective CD28 and ICOS during the course of an immune response exacerbated both Th1 and Th2 related diseases (1). Although originally ICOS was identified as a costimulatory molecule for Th2 cells, ICOS does not appear to act uniquely in Th2 cells. Rather, Th2 driven responses in general seem to be more dependent on costimulation than Th1 responses (73). Both CD28 and ICOS deficient mice have difficulties in forming functional germinal centers during infection with viruses or bacterial substances. Mice that are defective for the B7/CD28 pathway do not form GCs

and fail to induce substantial levels of IgG (2, 74, 75). Although primary germinal centers are formed in B7RP-1$^{-/-}$ or ICOS$^{-/-}$ mice, they are smaller in size than GCs formed in wild type mice and fail to develop into large secondary GCs (76, 77). This is accompanied by a reduction in serum IgG1 and IgG2a. Intestinal GCs, the Peyers patches, do develop in ICOS$^{-/-}$ mice, but also have a smaller diameter (78). The diminished capacity to drive a Th1 and Th2 mediated humoral response in the absence of ICOS and CD28 could either be a direct effect on Th differentiation or T:B cell help or indirectly be caused by the effect on T cell expansion. Most interestingly, as a result of disruptive germinal center reactions, ICOS-deficient patients suffer from severe hypo-gammaglobulemia, and disturbed B cell homeostasis leading to recurrent bacterial infections: a common variable immunodeficiency phenotype (68). T cell homeostasis seems not to be affected in these patients, but a decrease in IL-10 production may contribute to the poor germinal center formation. These data support a crucial role for ICOS in humoral responses.

Regulatory T cells

The importance of CD28 and ICOS during an *in vivo* immune response could also arise by their function in a relatively small subset of peripheral T cells, called regulatory T cells (Treg). This subset of T cells not only is indispensable for the maintenance of T cell tolerance against self-Ags but also suppresses immune responses against microbes invading or cohabiting the host (reviewed in Refs. 79, 80). Recently, compelling evidence arose for a role of CD28 in the development of regulatory T cells. CD28 is essential in the development and maintenance of regulatory T cells by the induction of regulatory T cell related genes FoxP3 and GITR in thymocytes (81-84). Regulatory T cells that are defective for CD28 have a diminished regulatory function, of which the residual function was demonstrated to be dependent on ICOS in an experimental colitis model (85). Moreover, ICOS expression is high on regulatory T cells and block of ICOS function on these cells abrogates their regulatory capacity in diabetic lesions (35), and an allergic asthma model (34), possibly through lack of inhibitory IL-10 production. So, although CD28 is mandatory for Treg development in the thymus, both CD28 and ICOS function in the periphery to maintain regulatory T cell function.

ICOS versus CD28 in immunotherapy

B7-costimulatory molecules form attractive targets for immunotherapy because they can be used either to enhance or inhibit antigen specific T cell responses. Blocking either CD28 or ICOS, or both of these costimulatory molecules can be beneficial when inhibition of aberrant immune activation is desired. *In vivo* block of B7:CD28 interaction has been shown to prolong graft survival and suppress autoimmunity as was also observed when ICOS: B7RP-1 interaction was inhibited (reviewed in Refs. 1 and 86). One should be cautious, however, since the dual role for both costimulators in activation of conventional CD4^{+} T cells and activation of suppressive Treg may have opposite effects, potentially resulting in the induction of even greater T cell activation (84, 87, 88). Vice versa, boosting the immune response via either CD28 or ICOS could have a

potentiating effect on otherwise insufficient immune responses such as anti-tumor responses or responses in immune-compromised individuals. For instance, vaccine non-responders could benefit from enhanced CD28 or ICOS-mediated costimulation.

Recently, another level of immune activation was found for CD28:B7 interactions. Agonistic signals through B7 on DCs could reduce suppressive Treg activity, presumably via IL-6 induction (89). The choice of targeting ICOS or CD28 may be influenced by the pathogenic T cell population involved. Since a subset of human memory T cells does not express CD28, targeting memory cells through ICOS is more favorable, while naïve T cells benefit more from CD28 directed targeting. In conclusion, with respect to immunotherapy both CD28 and ICOS are promising targets each having a unique applicability and potential depending on the T cell subset targeted.

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References

1. Greenwald, R. J., G. J. Freeman, and A. H. Sharpe. 2005. The B7 family revisited. *Annu.Rev.Immunol.* 23:515-548.
2. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261:609-612.
3. Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1:405-413.
4. McAdam, A. J., R. J. Greenwald, M. A. Levin, T. Chernova, N. Malenkovich, V. Ling, G. J. Freeman, and A. H. Sharpe. 2001. ICOS is critical for CD40-mediated antibody class switching. *Nature* 409:102-105.
5. Tafuri, A., A. Shahinian, F. Bladt, S. K. Yoshinaga, M. Jordana, A. Wakeham, L. M. Boucher, D. Bouchard, V. S. Chan, G. Duncan, B. Odermatt, A. Ho, A. Itie, T. Horan, J. S. Whoriskey, T. Pawson, J. M. Penninger, P. S. Ohashi, and T. W. Mak. 2001. ICOS is essential for effective T-helper-cell responses. *Nature* 409:105-109.
6. Dong, C., A. E. Juedes, U. A. Temann, S. Shresta, J. P. Allison, N. H. Ruddle, and R. A. Flavell. 2001. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 409:97-101.
7. Dong, H., G. Zhu, K. Tamada, and L. Chen. 1999. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat.Med.* 5:1365-1369.
8. Freeman, G. J., A. J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L. J. Fitz, N. Malenkovich, T. Okazaki, M. C. Byrne, H. F. Horton, L. Fouser, L. Carter, V. Ling, M. R. Bowman, B. M. Carreno, M. Collins, C. R. Wood, and T. Honjo. 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J.Exp.Med.* 192:1027-1034.
9. Latchman, Y., C. R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A. J. Long, J. A. Brown, R. Nunes, E. A. Greenfield, K. Bourque, V. A. Boussiotis, L. L. Carter, B. M. Carreno, N. Malenkovich, H. Nishimura, T. Okazaki, T. Honjo, A. H. Sharpe, and G. J. Freeman. 2001. PD-L2 is a

second ligand for PD-1 and inhibits T cell activation. *Nat.Immunol.* 2:261-268.

10. Watanabe, N., M. Gavrieli, J. R. Sedy, J. Yang, F. Fallarino, S. K. Loftin, M. A. Hurchla, N. Zimmerman, J. Sim, X. Zang, T. L. Murphy, J. H. Russell, J. P. Allison, and K. M. Murphy. 2003. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat.Immunol.* 4:670-679.
11. Chapoval, A. I., J. Ni, J. S. Lau, R. A. Wilcox, D. B. Flies, D. Liu, H. Dong, G. L. Sica, G. Zhu, K. Tamada, and L. Chen. 2001. B7-H3: a costimulatory molecule for T cell activation and IFN-γ production. *Nat.Immunol.* 2:269-274.
12. Sica, G. L., I. H. Choi, G. Zhu, K. Tamada, S. D. Wang, H. Tamura, A. I. Chapoval, D. B. Flies, J. Bajorath, and L. Chen. 2003. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. *Immunity* 18:849-861.
13. Prasad, D. V., S. Richards, X. M. Mai, and C. Dong. 2003. B7S1, a novel B7 family member that negatively regulates T cell activation. *Immunity* 18:863-873.
14. Suh, W. K., B. U. Gajewska, H. Okada, M. A. Gronski, E. M. Bertram, W. Dawicki, G. S. Duncan, J. Bukczynski, S. Plyte, A. Elia, A. Wakeham, A. Itie, S. Chung, J. Da Costa, S. Arya, T. Horan, P. Campbell, K. Gaida, P. S. Ohashi, T. H. Watts, S. K. Yoshinaga, M. R. Bray, M. Jordana, and T. W. Mak. 2003. The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses. *Nat.Immunol.* 4:899-906.
15. Watts, T. H. and M. A. DeBenedette. 1999. T cell co-stimulatory molecules other than CD28. *Curr.Opin.Immunol.* 11:286-293.
16. Rogers, P. R. and M. Croft. 2000. CD28, Ox-40, LFA-1, and CD4 modulation of Th1/Th2 differentiation is directly dependent on the dose of antigen. *J.Immunol.* 164:2955-2963.
17. Watts, T. H. 2005. TNF/TNFR family members in costimulation of T cell responses. *Annu.Rev.Immunol.* 23:23-68.
18. Yoshinaga, S. K., J. S. Whoriskey, S. D. Khare, U. Sarmiento, J. Guo, T. Horan, G. Shih, M. Zhang, M. A. Coccia, T. Kohno, A. Tafuri-Bladt, D. Brankow, P. Campbell, D. Chang, L. Chiu, T. Dai, G. Duncan, G. S. Elliott, A. Hui, S. M. McCabe, S. Scully, A. Shahinian, C. L. Shaklee, G. Van, T. W. Mak, and G. Senaldi. 1999. T-cell co-stimulation through B7RP-1 and ICOS. *Nature* 402:827-832.
19. Hutloff, A., A. M. Dittrich, K. C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos, and R. A. Kroczek. 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 397:263-266.
20. Harper, K., C. Balzano, E. Rouvier, M. G. Mattei, M. F. Luciani, and P. Golstein. 1991. CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. *J.Immunol.* 147:1037-1044.
21. Mages, H. W., A. Hutloff, C. Heuck, K. Buchner, H. Himmelbauer, F. Oliveri, and R. A. Kroczek. 2000. Molecular cloning and characterization of murine ICOS and identification of B7h as ICOS ligand. *Eur.J.Immunol.* 30:1040-1047.
22. Beier, K. C., A. Hutloff, A. M. Dittrich, C. Heuck, A. Rauch, K. Buchner, B. Ludewig, H. D. Ochs, H. W. Mages, and R. A. Kroczek. 2000. Induction, binding specificity and function of human ICOS. *Eur.J.Immunol.* 30:3707-3717.
23. Suh, W. K., A. Tafuri, N. N. Berg-Brown, A. Shahinian, S. Plyte, G. S. Duncan, H. Okada, A. Wakeham, B. Odermatt, P. S. Ohashi, and T. W. Mak. 2004. The inducible costimulator plays the major costimulatory role in humoral immune responses in the absence of CD28. *J.Immunol.* 172:5917-5923.
24. Appay, V., J. J. Zaunders, L. Papagno, J. Sutton, A. Jaramillo, A. Waters, P. Easterbrook, P. Grey, D. Smith, A. J. McMichael, D. A. Cooper, S. L. Rowland-

Jones, and A. D. Kelleher. 2002. Characterization of CD4+ CTLs *ex vivo*. *J.Immunol.* 168:5954-5958.

25. Amyes, E., A. J. McMichael, and M. F. Callan. 2005. Human CD4+ T Cells Are Predominantly Distributed among Six Phenotypically and Functionally Distinct Subsets. *J.Immunol.* 175:5765-5773.
26. McAdam, A. J., T. T. Chang, A. E. Lumelsky, E. A. Greenfield, V. A. Boussiotis, J. S. Duke-Cohan, T. Chernova, N. Malenkovich, C. Jabs, V. K. Kuchroo, V. Ling, M. Collins, A. H. Sharpe, and G. J. Freeman. 2000. Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells. *J.Immunol.* 165:5035-5040.
27. Coyle, A. J., S. Lehar, C. Lloyd, J. Tian, T. Delaney, S. Manning, T. Nguyen, T. Burwell, H. Schneider, J. A. Gonzalo, M. Gosselin, L. R. Owen, C. E. Rudd, and J. C. Gutierrez-Ramos. 2000. The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. *Immunity* 13:95-105.
28. Vieira, P. L., L. Wassink, L. M. Smith, S. Nam, G. A. Kingsbury, J. C. Gutierrez-Ramos, A. J. Coyle, M. L. Kapsenberg, and E. A. Wierenga. 2004. ICOS-mediated signaling regulates cytokine production by human T cells and provides a unique signal to selectively control the clonal expansion of Th2 helper cells. *Eur.J.Immunol.* 34:1282-1290.
29. Wassink, L., P. L. Vieira, H. H. Smits, G. A. Kingsbury, A. J. Coyle, M. L. Kapsenberg, and E. A. Wierenga. 2004. ICOS expression by activated human Th cells is enhanced by IL-12 and IL-23: increased ICOS expression enhances the effector function of both Th1 and Th2 cells. *J.Immunol.* 173:1779-1786.
30. Yagi, J., Y. Arimura, U. Dianzani, T. Uede, T. Okamoto, and T. Uchiyama. 2003. Regulatory roles of IL-2 and IL-4 in H4/inducible costimulator expression on activated CD4+ T cells during Th cell development. *J.Immunol.* 171:783-794.
31. van Berkel, M. E., E. H. Schrijver, F. M. Hofhuis, A. H. Sharpe, A. J. Coyle, C. P. Broeren, K. Tesselaar, and M. A. Oosterwegel. 2005. ICOS contributes to T cell expansion in CTLA-4 deficient mice. *J.Immunol.* 175:182-188.
32. Villegas, E. N., L. A. Lieberman, N. Mason, S. L. Blass, V. P. Zediak, R. Peach, T. Horan, S. Yoshinaga, and C. A. Hunter. 2002. A role for inducible costimulator protein in the CD28- independent mechanism of resistance to *Toxoplasma gondii*. *J.Immunol.* 169:937-943.
33. Lohning, M., A. Hutloff, T. Kallinich, H. W. Mages, K. Bonhagen, A. Radbruch, E. Hamelmann, and R. A. Kroczek. 2003. Expression of ICOS *in vivo* defines CD4+ effector T cells with high inflammatory potential and a strong bias for secretion of interleukin 10. *J.Exp.Med.* 197:181-193.
34. Akbari, O., G. J. Freeman, E. H. Meyer, E. A. Greenfield, T. T. Chang, A. H. Sharpe, G. Berry, R. H. DeKruyff, and D. T. Umetsu. 2002. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyper-reactivity. *Nat.Med.* 8:1024-1032.
35. Herman, A. E., G. J. Freeman, D. Mathis, and C. Benoist. 2004. CD4+CD25+ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. *J.Exp.Med.* 199:1479-1489.
36. Azuma, M., D. Ito, H. Yagita, K. Okumura, J. H. Phillips, L. L. Lanier, and C. Somoza. 1993. B70 antigen is a second ligand for CTLA-4 and CD28. *Nature* 366:76-79.
37. Freeman, G. J., J. G. Gribben, V. A. Boussiotis, J. W. Ng, V. A. Restivo, Jr., L. A. Lombard, G. S. Gray, and L. M. Nadler. 1993. Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. *Science* 262:909-911.
38. Hathcock, K. S., G. Laszlo, C. Pucillo, P. Linsley, and R. J. Hodes. 1994. Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. *J.Exp.Med.*

180:631-640.

39. Zhou, Z., K. Hoebe, X. Du, Z. Jiang, L. Shamel, and B. Beutler. 2005. Antagonism between MyD88- and TRIF-dependent signals in B7RP-1 up-regulation. *Eur.J.Immunol.* 35:1918-1927.
40. Aicher, A., M. Hayden-Ledbetter, W. A. Brady, A. Pezzutto, G. Richter, D. Magaletti, S. Buckwalter, J. A. Ledbetter, and E. A. Clark. 2000. Characterization of human inducible costimulator ligand expression and function. *J.Immunol.* 164:4689-4696.
41. Liang, L., E. M. Porter, and W. C. Sha. 2002. Constitutive expression of the B7h ligand for inducible costimulator on naïve B cells is extinguished after activation by distinct B cell receptor and interleukin 4 receptor-mediated pathways and can be rescued by CD40 signaling. *J.Exp.Med.* 196:97-108.
42. Wang, S., G. Zhu, A. I. Chapoval, H. Dong, K. Tamada, J. Ni, and L. Chen. 2000. Costimulation of T cells by B7-H2, a B7-like molecule that binds ICOS. *Blood* 96:2808-2813.
43. Swallow, M. M., J. J. Wallin, and W. C. Sha. 1999. B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha. *Immunity* 11:423-432.
44. Khayyamian, S., A. Hutloff, K. Buchner, M. Grafe, V. Henn, R. A. Kroczek, and H. W. Mages. 2002. ICOS-ligand, expressed on human endothelial cells, costimulates Th1 and Th2 cytokine secretion by memory $CD4^+$ T cells. *Proc.Natl.Acad.Sci.U.S.A.* 99:6198-6203.
45. Diehn, M., A. A. Alizadeh, O. J. Rando, C. L. Liu, K. Stankunas, D. Botstein, G. R. Crabtree, and P. O. Brown. 2002. Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation. *Proc.Natl.Acad.Sci.U.S.A.* 99:11796-11801.
46. Riley, J. L., M. Mao, S. Kobayashi, M. Biery, J. Burchard, G. Cavet, B. P. Gregson, C. H. June, and P. S. Linsley. 2002. Modulation of TCR-induced transcriptional profiles by ligation of CD28, ICOS, and CTLA-4 receptors. *Proc.Natl.Acad.Sci.U.S.A.* 99:11790-11795.
47. Acuto, O. and F. Michel. 2003. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat.Rev.Immunol.* 3:939-951.
48. Rudd, C. E. and H. Schneider. 2003. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. *Nat.Rev.Immunol.* 3:544-556.
49. Pages, F., M. Ragueneau, R. Rottapel, A. Truneh, J. Nunes, J. Imbert, and D. Olive. 1994. Binding of phosphatidylinositol-3-OH kinase to CD28 is required for T-cell signalling. *Nature* 369:327-329.
50. Okkenhaug, K., L. Wu, K. M. Garza, J. La Rose, W. Khoo, B. Odermatt, T. W. Mak, P. S. Ohashi, and R. Rottapel. 2001. A point mutation in CD28 distinguishes proliferative signals from survival signals. *Nat.Immunol.* 2:325-332.
51. Kim, H. H., M. Tharayil, and C. E. Rudd. 1998. Growth factor receptor-bound protein 2 SH2/SH3 domain binding to CD28 and its role in co-signaling. *J.Biol.Chem.* 273:296-301.
52. Chuang, E., T. S. Fisher, R. W. Morgan, M. D. Robbins, J. M. Duerr, M. G. Vander Heiden, J. P. Gardner, J. E. Hambor, M. J. Neveu, and C. B. Thompson. 2000. The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A. *Immunity* 13:313-322.
53. Holdorf, A. D., J. M. Green, S. D. Levin, M. F. Denny, D. B. Straus, V. Link, P. S. Changelian, P. M. Allen, and A. S. Shaw. 1999. Proline residues in CD28 and the Src homology (SH)3 domain of Lck are required for T cell costimulation. *J.Exp.Med.* 190:375-384.
54. Raab, M., Y. C. Cai, S. C. Bunnell, S. D. Heyeck, L. J. Berg, and C. E. Rudd. 1995. p56Lck and p59Fyn regulate CD28 binding to phosphatidylinositol 3-kinase, growth factor receptor-bound protein GRB-2, and T cell-specific protein-tyrosine kinase ITK:

implications for T-cell costimulation. *Proc.Natl.Acad.Sci.U.S.A.* 92:8891-8895.

55. Gibson, S., K. Truitt, Y. Lu, R. Lapushin, H. Khan, J. B. Imboden, and G. B. Mills. 1998. Efficient CD28 signalling leads to increases in the kinase activities of the TEC family tyrosine kinase EMT/ITK/TSK and the SRC family tyrosine kinase LCK. *Biochem.J.* 330:1123-1128.
56. Miller, A. T. and L. J. Berg. 2002. New insights into the regulation and functions of Tec family tyrosine kinases in the immune system. *Curr.Opin.Immunol.* 14:331-340.
57. Andres, P. G., K. C. Howland, A. Nirula, L. P. Kane, L. Barron, D. Dresnek, A. Sadra, J. Imboden, A. Weiss, and A. K. Abbas. 2004. Distinct regions in the CD28 cytoplasmic domain are required for T helper type 2 differentiation. *Nat.Immunol.* 5:435-442.
58. Harada, Y., D. Ohgai, R. Watanabe, K. Okano, O. Koiwai, K. Tanabe, H. Toma, A. Altman, and R. Abe. 2003. A single amino acid alteration in cytoplasmic domain determines IL-2 promoter activation by ligation of CD28 but not inducible costimulator (ICOS). *J.Exp.Med.* 197:257-262.
59. Suzuki, Y., C. Demoliere, D. Kitamura, H. Takeshita, U. Deuschle, and T. Watanabe. 1997. HAX-1, a novel intracellular protein, localized on mitochondria, directly associates with HS1, a substrate of Src family tyrosine kinases. *J.Immunol.* 158:2736-2744.
60. Arimura, Y., H. Kato, U. Dianzani, T. Okamoto, S. Kamekura, D. Buonfiglio, T. Miyoshi-Akiyama, T. Uchiyama, and J. Yagi. 2002. A co-stimulatory molecule on activated T cells, H4/ICOS, delivers specific signals in T(h) cells and regulates their responses. *Int.Immunol.* 14:555-566.
61. Su, B., E. Jacinto, M. Hibi, T. Kallunki, M. Karin, and Y. Ben Neriah. 1994. JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* 77:727-736.
62. Green, J. M., P. J. Noel, A. I. Sperling, T. L. Walunas, G. S. Gray, J. A. Bluestone, and C. B. Thompson. 1994. Absence of B7-dependent responses in CD28-deficient mice. *Immunity* 1:501-508.
63. Noel, P. J., L. H. Boise, J. M. Green, and C. B. Thompson. 1996. CD28 costimulation prevents cell death during primary T cell activation. *J.Immunol.* 157:636-642.
64. Nurieva, R. I., X. M. Mai, K. Forbush, M. J. Bevan, and C. Dong. 2003. B7h is required for T cell activation, differentiation, and effector function. *Proc.Natl.Acad.Sci.U.S.A.* 100:14163-14168.
65. Riley, J. L., P. J. Blair, J. T. Musser, R. Abe, K. Tezuka, T. Tsuji, and C. H. June. 2001. ICOS costimulation requires IL-2 and can be prevented by CTLA-4 engagement. *J.Immunol.* 166:4943-4948.
66. Sporici, R. A., R. L. Beswick, C. von Allmen, C. A. Rumbley, M. Hayden-Ledbetter, J. A. Ledbetter, and P. J. Perrin. 2001. ICOS ligand costimulation is required for T-cell encephalitogenicity. *Clin.Immunol.* 100:277-288.
67. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat.Immunol.* 6:1133-1141.
68. Warnatz, K., L. Bossaller, U. Salzer, A. Skrabl-Baumgartner, W. Schwinger, van der Burg M., J. J. van Dongen, M. Orlowska-Volk, R. Knoth, A. Durandy, R. Draeger, M. Schlesier, H. H. Peter, and B. Grimbacher. 2005. Human ICOS-deficiency abrogates the germinal center reaction and provides a monogenic model for common variable immunodeficiency. *Blood* 29: in press.
69. Harada, H., A. D. Salama, M. Sho, A. Izawa, S. E. Sandner, T. Ito, H. Akiba, H. Yagita, A. H. Sharpe, G. J. Freeman, and M. H. Sayegh. 2003. The role of the ICOS-B7h T cell costimulatory pathway in transplantation immunity. *J.Clin.Invest.* 112:234-243.
70. Smith, K. M., J. M. Brewer, P. Webb, A. J.

Coyle, C. Gutierrez-Ramos, and P. Garside. 2003. Inducible costimulatory molecule-B7-related protein 1 interactions are important for the clonal expansion and B cell helper functions of naïve, Th1, and Th2 T cells. *J.Immunol.* 170:2310-2315.

71. Gonzalo, J. A., T. Delaney, J. Corcoran, A. Goodearl, J. C. Gutierrez-Ramos, and A. J. Coyle. 2001. Cutting edge: the related molecules CD28 and inducible costimulator deliver both unique and complementary signals required for optimal T cell activation. *J.Immunol.* 166:1-5.
72. Guo, J., M. Stolina, J. V. Bready, S. Yin, T. Horan, S. K. Yoshinaga, and G. Senaldi. 2001. Stimulatory effects of B7-related protein-1 on cellular and humoral immune responses in mice. *J.Immunol.* 166:5578-5584.
73. Sperling, A. I. and J. A. Bluestone. 2001. ICOS costimulation: Its not just for TH2 cells anymore. *Nat.Immunol.* 2:573-574.
74. Borriello, F., M. P. Sethna, S. D. Boyd, A. N. Schweitzer, E. A. Tivol, D. Jacoby, T. B. Strom, E. M. Simpson, G. J. Freeman, and A. H. Sharpe. 1997. B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. *Immunity* 6:303-313.
75. Ferguson, S. E., S. Han, G. Kelsoe, and C. B. Thompson. 1996. CD28 is required for germinal center formation. *J.Immunol.* 156:4576-4581.
76. Dong, C., U. A. Temann, and R. A. Flavell. 2001. Cutting edge: critical role of inducible costimulator in germinal center reactions. *J.Immunol.* 166:3659-3662.
77. Mak, T. W., A. Shahinian, S. K. Yoshinaga, A. Wakeham, L. M. Boucher, M. Pintilie, G. Duncan, B. U. Gajewska, M. Gronski, U. Eriksson, B. Odermatt, A. Ho, D. Bouchard, J. S. Whorisky, M. Jordana, P. S. Ohashi, T. Pawson, F. Bladt, and A. Tafuri. 2003. Costimulation through the inducible costimulator ligand is essential for both T helper and B cell functions in T cell-dependent B cell responses. *Nat.Immunol.* 4:765-772.
78. Iiyama, R., T. Kanai, K. Uraushihara, T. Totsuka, T. Nakamura, T. Miyata, H. Yagita, A. Kushi, K. Suzuki, K. Tezuka, and M. Watanabe. 2003. The role of inducible co-stimulator (ICOS)/B7-related protein-1 (B7RP-1) interaction in the functional development of Peyer's patches. *Immunol.Lett.* 88:63-70.
79. Bluestone, J. A. and A. K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nat.Rev.Immunol.* 3:253-257.
80. Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 101:455-458.
81. Tai, X., M. Cowan, L. Feigenbaum, and A. Singer. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat.Immunol.* 6:152-162.
82. Lohr, J., B. Knoechel, E. C. Kahn, and A. K. Abbas. 2004. Role of B7 in T cell tolerance. *J.Immunol.* 173:5028-5035.
83. Tang, Q., K. J. Henriksen, E. K. Boden, A. J. Tooley, J. Ye, S. K. Subudhi, X. X. Zheng, T. B. Strom, and J. A. Bluestone. 2003. Cutting edge: CD28 controls peripheral homeostasis of $CD4^+CD25^+$ regulatory T cells. *J.Immunol.* 171:3348-3352.
84. Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12:431-440.
85. de Jong, Y. P., S. T. Rietdijk, W. A. Faubion, A. C. Abadia-Molina, K. Clarke, E. Mizoguchi, J. Tian, T. Delaney, S. Manning, J. C. Gutierrez-Ramos, A. K. Bhan, A. J. Coyle, and C. Terhorst. 2004. Blocking inducible co-stimulator in the absence of CD28 impairs Th1 and CD25+ regulatory T cells in murine colitis. *Int.Immunol.* 16:205-

213.

86. Salomon, B. and J. A. Bluestone. 2001. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu.Rev.Immunol.* 19:225-252.
87. Rottman, J. B., T. Smith, J. R. Tonra, K. Ganley, T. Bloom, R. Silva, B. Pierce, J. C. Gutierrez-Ramos, E. Ozkaynak, and A. J. Coyle. 2001. The costimulatory molecule ICOS plays an important role in the immunopathogenesis of EAE. *Nat.Immunol.* 2:605-611.
88. Racke, M. K., D. E. Scott, L. Quigley, G. S. Gray, R. Abe, C. H. June, and P. J. Perrin. 1995. Distinct roles for B7-1 (CD-80) and B7-2 (CD-86) in the initiation of experimental allergic encephalomyelitis. *J.Clin.Invest.* 96:2195-2203.
89. Orabona, C., U. Grohmann, M. L. Belladonna, F. Fallarino, C. Vacca, R. Bianchi, S. Bozza, C. Volpi, B. L. Salomon, M. C. Fioretti, L. Romani, and P. Puccetti. 2004. CD28 induces immunostimulatory signals in dendritic cells via CD80 and CD86. *Nat.Immunol.* 5:1134-1142.
90. Roepman, P., L. F. Wessels, N. Kettelarij, P. Kemmeren, A. J. Miles, P. Lijnzaad, M. G. Tilanus, R. Koole, G. J. Hordijk, P. C. van der Vliet, M. J. Reinders, P. J. Slootweg, and F. C. Holstege. 2005. An expression profile for diagnosis of lymph node metastases from primary head and neck squamous cell carcinomas. *Nat.Genet.* 37:182-186.
91. van de Peppel, J., P. Kemmeren, H. van Bakel, M. Radonjic, D. van Leenen, and F. C. Holstege. 2003. Monitoring global messenger RNA changes in externally controlled microarray experiments. *EMBO Rep.* 4:387-393.
92. Tusher, V. G., R. Tibshirani, and G. Chu. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc.Natl.Acad.Sci. U.S.A.* 98:5116-5121.

Chapter 2

Introduction to this thesis

B7-related costimulatory molecules

In addition to signals generated through MHC-Ag recognition by the TCR, T cells require additional costimulatory signals to become fully activated. The family of B7 costimulatory molecules is a group molecules belonging to the Ig-superfamily that consist of positive and negative costimulators of T cells (**Fig. 1**). The contribution of each of these costimulation ligand-receptor pair to either enhancement or suppression of a TCR-driven T cell response is discussed in more detail below.

Negative costimulators: CTLA-4, PD-1, BLTA

Negative costimulatory molecules can suppress T cell responses on two different levels: they either prevent unwanted T cell responses by maintaining a tolerogenic state in the T cell or they inhibit ongoing T cell responses once the Ag is cleared. CTLA-4 is by far the most potent attenuator of T cell responses (1, 2). As the inhibitory counterpart of CD28, it also associates with B7.1 and B7.2. Its expression is low on naïve T cells, is readily upregulated after activation (3), and is constitutive on regulatory T cells (4). Ligation of CTLA-4 can result in inhibition of T cell responses using several mechanisms: by direct interference with TCR signaling or costimulation signaling, by competing with CD28 for B7 ligand binding, and/or by delivering negative signals in the DC through B7.1/2 (5-7). The inhibitory function of CTLA-4 is most clearly seen in CTLA-4-deficient mice that display massive expansion of the T cell pool (8). The aberrant T cell activation in CTLA-4-deficient mice might be caused by two different mechanisms. First, CTLA-4$^{-/-}$ T cells hyperproliferate in the absence of its inhibitory signals (9, 10). In addition, the lack of CTLA-4 could release tolerogenic signals in the naïve T cell to self-antigens, which is either cell intrinsic or by means of CTLA-4 function on suppressive regulatory T cells. Not only is CTLA-4 highly expressed on this subset, it proved to be essential for execution of the suppressive effect of Treg on pathogenic T cells (4, 11).

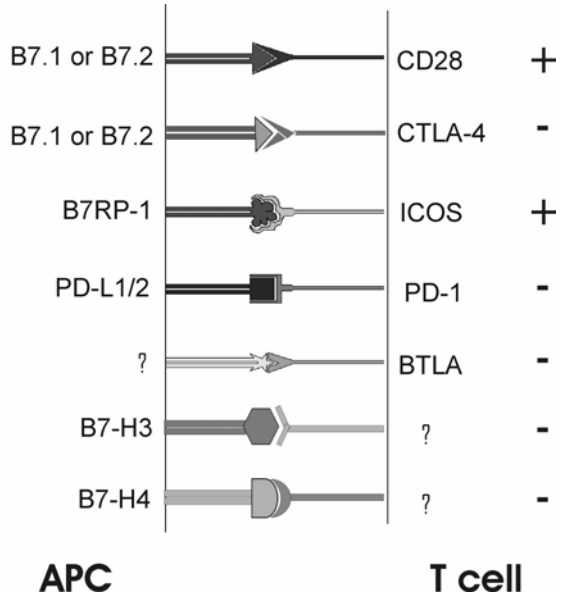

Figure 1. Schematic representation of members of the B7-costimulatory family. B7-ligands are expressed on the APC and their respective CD28-like receptors on the T cells. Indicated are the signals generated in the T cell after interaction of the receptor with its respective ligand.

Compared to CTLA-4, PD-1 (programmed cell death 1) is a less potent inhibitor of T cell activation. Unlike other CD28 homologues PD-1 expression is not limited to activated T cells but is also present on recently stimulated B cells and myeloid cells (12). Two ligands have been identified for PD-1; PD-L1, which is present on a diversity of cell types (including cells of non-haematopoietic origin) and PD-L2 of which the expression pattern is limited to

DCs and a subset of macrophages (13-15). Depending on the mouse strain, mice deficient for PD-1 exhibit mild splenomegaly and develop spontaneous autoimmune disorders after a period of time, like arthritis and glomerulonephritis (C57/Bl6) or (16) the more acute cardiomyopathy and can accelerate onset of diabetes in NOD mice (17-19). Recently, an important attenuating function of PD-1 on viral specific $CD8^+$ T cells during chronic LCMV infection was described, implicating a specific function of PD-1 on $CD8^+$ T cells (20).

The last negative regulatory receptor of this family discovered so far is called B and T lymphocyte attenuator (BLTA), reflected molecularly by a two ITIM containing cytoplasmic tail (21). The expression of BLTA is induced after activation, persists during differentiation and is maintained only on Th1-differentiated T cells. Recently, a non-B7 like molecule, herpus virus entry mediator, (HVEM) was identified as a ligand for BLTA (22). HVEM is expressed on resting T cells and downregulated upon activation. *In vitro*, T cells lacking BLTA show enhanced proliferation compared to wild type T cells. Like CTLA-$4^{-/-}$ and PD-$1^{-/-}$ mice, mice deficient for BLTA are more susceptible to the induction of auto-immune disease (21). The relative contribution of BLTA compared to the other negative regulators of T cell activation remains to be determined.

For two novel B7-like ligands, B7-H3 and B7-H4 (B7x, B7S1), which attenuate T cell function, no counterreceptor has been identified yet. B7-H4 is broadly expressed, with expression on professional APC as well as on non-lymphoid tissue (23, 24). In contrast, B7-H3 expression is restricted to activated DCs and monocytes (25-27). Their counter-receptor is not present on naïve T cells, but is upregulated after activation. Discovering its ligand(s) and expression pattern will shed more light on the regulation of this receptor ligand-pair and the relative contribution to inhibition of T cell activation compared to BLTA, PD-1 and CTLA-4.

Positive costimulators

Since the cloning of CD28, only ICOS as a CD28 homologue with positive costimulator activity has been identified (both discussed in **chapter 1**). However, several clues in literature point to additional positive regulatory molecules, yet to be identified.

First, next to the negative signal via PD-1 ligation, both PDL-1 and PDL-2 can also positively costimulate T cells in terms of T cell proliferation and mainly IL-10 and IFN-γ production, respectively (14, 28, 29). More substantial evidence was provided by Wang *et al.*, who demonstrated that both ligands mutated for the PD-1 binding domain could still activate T cells (30). In fact, T cells deficient for PD-1 remained sensitive to PDL-1 and PDL-2 stimulation (29, 30). Whether indeed a positive counterpart of PD-1 exists, similar to CD28 and CTLA-4, remains to be determined.

Second, several laboratories have provided evidence for a novel CD28-like molecule, with binding capacity to B7.1 and B7.2. Not only could CD28-CTLA-$4^{-/-}$ T cells be stimulated with B7 *in vitro*, also B7 blockade in mice deficient for both CD28 and CTLA-4 significantly prolonged graft survival (31, 32). Moreover, although blockade of CD28 could inhibit generation

of Ag-specific T cells *in vivo*, blocking both B7.1/2 simultaneously was more effective (33). This strongly indicates the existence of a third unidentified receptor that binds B7.1 or B7.2. Also, this third receptor might be more important in later stages of the immune response, since secondary responses in terms of Ab production was much more diminished by the block of B7.1/2 compared to the block of CD28 (33).

Scope of this thesis

It is well established that CD28- and ICOS-mediated costimulation are crucial for an optimal T cell dependent immune response. Within the family of B7 costimulatory molecules, ICOS resembles CD28 function the most (discussed in detail in **chapter 1**). This thesis addresses the differential function of CD28 and in the induction of $CD4^+$ T cell expansion, survival and B cell help. In **chapter 3** the role for ICOS in clonal expansion of T cells in an *in vivo* T cell proliferation model, the CTLA-$4^{-/-}$ mice is investigated. It is shown that next to CD28, ICOS provides important signals for the expansion of the murine $CD4^+$ T cell pool *in vivo* and *in vitro*. In **chapter 4** evidence is provided for ICOS-induced cell survival in human $CD4^+$ T cells via mechanisms similar to those induced by CD28. A possible new intracellular binding partner for both CD28 and ICOS is discussed. Lastly, the role of ICOS and CD28 in the generation of protective Abs is addressed in an *in vivo* vaccination model in **chapter 5**. We have demonstrated that ICOS- and CD28 together account for the adjuvant activity of wild type H44/76 LPS and less toxic variant *lpxL1* LPS in terms of *N. meningitidis*-specific IgG production. Moreover, ICOS-mediated costimulation predominates in the adjuvant activity of *lpxL1* LPS compared to CD28-mediated costimulation. In **chapter 6** the contribution of a putative B7.1/2 binding CD28 homologue in an allergic airway hypersensitivity model is explored. In the last chapter, **chapter 7** the implication of our findings are discussed in the context of a T cell dependent immune response.

References

1. Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1:405-413.
2. Krummel, M. F. and J. P. Allison. 1995. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J.Exp.Med.* 182:459-465.
3. Perkins, D., Z. Wang, C. Donovan, H. He, D. Mark, G. Guan, Y. Wang, T. Walunas, J. Bluestone, J. Listman, and P. W. Finn. 1996. Regulation of CTLA-4 expression during T cell activation. *J.Immunol.* 156:4154-4159.
4. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by $CD25^+$ $CD4^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J.Exp.Med.* 192:303-310.
5. Brunner, M. C., C. A. Chambers, F. K. Chan, J. Hanke, A. Winoto, and J. P. Allison. 1999. CTLA-4-Mediated inhibition of early events of T cell proliferation. *J.Immunol.* 162:5813-5820.
6. Grohmann, U., C. Orabona, F. Fallarino, C. Vacca, F. Calcinaro, A. Falorni, P. Candeloro, M. L. Belladonna, R. Bianchi, M. C. Fioretti, and P. Puccetti. 2002. CTLA-4-Ig regulates tryptophan catabolism *in vivo*. *Nat.Immunol.* 3:1097-1101.
7. Riley, J. L., M. Mao, S. Kobayashi, M. Biery, J. Burchard, G. Cavet, B. P. Gregson, C. H. June, and P. S. Linsley. 2002. Modulation of

TCR-induced transcriptional profiles by ligation of CD28, ICOS, and CTLA-4 receptors. *Proc.Natl.Acad.Sci.U.S.A.* 99:11790-11795.

8. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541-547.
9. Thompson, C. B. and J. P. Allison. 1997. The emerging role of CTLA-4 as an immune attenuator. *Immunity* 7:445-450.
10. Allison, J. P. and M. F. Krummel. 1995. The Yin and Yang of T cell costimulation. *Science* 270:932-933.
11. Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD25^{+}CD4^{+}$ regulatory cells that control intestinal inflammation. *J.Exp.Med.* 192:295-302.
12. Agata, Y., A. Kawasaki, H. Nishimura, Y. Ishida, T. Tsubata, H. Yagita, and T. Honjo. 1996. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int.Immunol.* 8:765-772.
13. Freeman, G. J., A. J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L. J. Fitz, N. Malenkovich, T. Okazaki, M. C. Byrne, H. F. Horton, L. Fouser, L. Carter, V. Ling, M. R. Bowman, B. M. Carreno, M. Collins, C. R. Wood, and T. Honjo. 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J.Exp.Med.* 192:1027-1034.
14. Dong, H., G. Zhu, K. Tamada, and L. Chen. 1999. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat.Med.* 5:1365-1369.
15. Latchman, Y., C. R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A. J. Long, J. A. Brown, R. Nunes, E. A. Greenfield, K. Bourque, V. A. Boussiotis, L. L. Carter, B. M. Carreno, N. Malenkovich, H. Nishimura, T. Okazaki, T. Honjo, A. H. Sharpe, and G. J. Freeman. 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat.Immunol.* 2:261-268.
16. Nishimura, H., M. Nose, H. Hiai, N. Minato, and T. Honjo. 1999. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 11:141-151.
17. Nishimura, H., N. Minato, T. Nakano, and T. Honjo. 1998. Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. *Int.Immunol.* 10:1563-1572.
18. Nishimura, H., T. Okazaki, Y. Tanaka, K. Nakatani, M. Hara, A. Matsumori, S. Sasayama, A. Mizoguchi, H. Hiai, N. Minato, and T. Honjo. 2001. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 291:319-322.
19. Wang, J., T. Yoshida, F. Nakaki, H. Hiai, T. Okazaki, and T. Honjo. 2005. Establishment of NOD-$Pdcd1^{-/-}$ mice as an efficient animal model of type I diabetes. *Proc.Natl.Acad.Sci.U.S.A.* 102:11823-11828.
20. Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman, and R. Ahmed. 2005. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 28:1-6.
21. Watanabe, N., M. Gavrieli, J. R. Sedy, J. Yang, F. Fallarino, S. K. Loftin, M. A. Hurchla, N. Zimmerman, J. Sim, X. Zang, T. L. Murphy, J. H. Russell, J. P. Allison, and K. M. Murphy. 2003. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat.Immunol.* 4:670-679.
22. Sedy, J. R., M. Gavrieli, K. G. Potter, M. A. Hurchla, R. C. Lindsley, K. Hildner, S. Scheu, K. Pfeffer, C. F. Ware, T. L. Murphy, and K. M. Murphy. 2005. B and T lymphocyte attenuator regulates T cell activation through interaction with

herpesvirus entry mediator. *Nat.Immunol.* 6:90-98.

23. Sica, G. L., I. H. Choi, G. Zhu, K. Tamada, S. D. Wang, H. Tamura, A. I. Chapoval, D. B. Flies, J. Bajorath, and L. Chen. 2003. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. *Immunity* 18:849-861.
24. Prasad, D. V., S. Richards, X. M. Mai, and C. Dong. 2003. B7S1, a novel B7 family member that negatively regulates T cell activation. *Immunity* 18:863-873.
25. Prasad, D. V., T. Nguyen, Z. Li, Y. Yang, J. Duong, Y. Wang, and C. Dong. 2004. Murine B7-H3 is a negative regulator of T cells. *J.Immunol.* 173:2500-2506.
26. Chapoval, A. I., J. Ni, J. S. Lau, R. A. Wilcox, D. B. Flies, D. Liu, H. Dong, G. L. Sica, G. Zhu, K. Tamada, and L. Chen. 2001. B7-H3: a costimulatory molecule for T cell activation and IFN-γ production. *Nat.Immunol.* 2:269-274.
27. Suh, W. K., B. U. Gajewska, H. Okada, M. A. Gronski, E. M. Bertram, W. Dawicki, G. S. Duncan, J. Bukczynski, S. Plyte, A. Elia, A. Wakeham, A. Itie, S. Chung, J. Da Costa, S. Arya, T. Horan, P. Campbell, K. Gaida, P. S. Ohashi, T. H. Watts, S. K. Yoshinaga, M. R. Bray, M. Jordana, and T. W. Mak. 2003. The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses. *Nat.Immunol.* 4:899-906.
28. Tseng, S. Y., M. Otsuji, K. Gorski, X. Huang, J. E. Slansky, S. I. Pai, A. Shalabi, T. Shin, D. M. Pardoll, and H. Tsuchiya. 2001. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J.Exp.Med.* 193:839-846.
29. Shin, T., G. Kennedy, K. Gorski, H. Tsuchiya, H. Koseki, M. Azuma, H. Yagita, L. Chen, J. Powell, D. Pardoll, and F. Housseau. 2003. Cooperative B7-1/2 (CD80/CD86) and B7-DC costimulation of $CD4^+$ T cells independent of the PD-1 receptor. *J.Exp.Med.* 198:31-38.
30. Wang, S., J. Bajorath, D. B. Flies, H. Dong, T. Honjo, and L. Chen. 2003. Molecular modeling and functional mapping of B7-H1 and B7-DC uncouple costimulatory function from PD-1 interaction. *J.Exp.Med.* 197:1083-1091.
31. Yamada, A., K. Kishimoto, V. M. Dong, M. Sho, A. D. Salama, N. G. Anosova, G. Benichou, D. A. Mandelbrot, A. H. Sharpe, L. A. Turka, H. Auchincloss, Jr., and M. H. Sayegh. 2001. CD28-independent costimulation of T cells in alloimmune responses. *J.Immunol.* 167:140-146.
32. Mandelbrot, D. A., M. A. Oosterwegel, K. Shimizu, A. Yamada, G. J. Freeman, R. N. Mitchell, M. H. Sayegh, and A. H. Sharpe. 2001. B7-dependent T-cell costimulation in mice lacking CD28 and CTLA4. *J.Clin.Invest.*107:881-887.
33. Haspot, F., F. Villemain, G. Laflamme, F. Coulon, D. Olive, J. Tiollier, J. P. Soulillou, and B. Vanhove. 2002. Differential effect of CD28 versus B7 blockade on direct pathway of allorecognition and self-restricted responses. *Blood* 99:2228-2234.

Chapter 3

ICOS contributes to T cell expansion in CTLA-4 deficient mice

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Summary

Both CD28 and Inducible Costimulator (ICOS) are important costimulatory molecules that promote Ag-specific cellular and humoral immune reactions. Whereas CD28 is generally thought to be the most important molecule in the initiation of a T cell response, ICOS is considered to act during the effector phase. We have investigated the contribution of ICOS to T cell responses in the absence of CTLA-4 mediated inhibition. Mice lacking CTLA-4, which show spontaneous CD28 mediated $CD4^+$ T cell activation, expansion and differentiation, were treated with antagonistic αICOS antibodies. Blocking the interaction between ICOS and its ligand B7RP-1 significantly reduced this aberrant T cell activation and caused a reduction in T cell numbers. *In vitro* analysis of $CD4^+$ T cells from treated mice revealed that ICOS blockade significantly reduced Th1 differentiation, while Th2 differentiation was only moderately inhibited. Further *in vitro* stimulation experiments demonstrated that ICOS is able to induce proliferation of murine $CD4^+$ and $CD8^+$ T cells, but only in the presence of IL-2. These results indicate that ICOS is not only important for T cell effector function, but also contributes to the expansion phase of a T cell response in the presence of CD28 signaling.

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Introduction

For an optimal T cell response a combination of signals generated by the Ag-specific T cell receptor (TCR) and costimulatory molecules is required. One of these is CD28, which is considered to be one of the most important costimulatory molecules during the onset of a T cell response. Ligation of CD28 with its ligands B7.1 and B7.2 can initiate T cell activation and production of IL-2 (reviewed in refs. 1, 2). Mice deficient for CD28 have disrupted primary T cell responses, which is reflected by reduced proliferative capacity, and a higher sensitivity to cell death (3-5). In addition, CD28 is important for $CD4^+$ T cell differentiation toward Th2, as shown in several disease models (reviewed in refs. 1, 2, 6). Although the requirement for CD28 in $CD8^+$ T cell responses seems to be less profound, defects in antiviral responses have been observed in the absence of CD28 (3, 7-9).

Similar functions have been described for the CD28 homologue ICOS (10-15). However, CD28 and ICOS differ in kinetics of expression, such that CD28 is constantly expressed on all T lymphocytes, whereas ICOS is enhanced after T cell activation (10, 11, 16). Like CD28, ICOS is required for both Th1 and Th2 development, being most indispensable in ongoing immune responses (17-21). This, together with the fact that ICOS triggering does not induce IL-2 production (11, 22), has led to the idea that ICOS is mainly important during the effector phase of the T cell response. Yet, there is evidence that ICOS can also support expansion of $CD4^+$ and $CD8^+$ T cells. *In vitro*, co-crosslinking the TCR and ICOS initiates $CD4^+$ T cell activation, as monitored by the induction of T cell proliferation (10, 11). Furthermore, primary $CD4^+$ T cell responses are diminished in the absence of ICOS signaling (14, 23). In addition, continuous stimulation of ICOS causes an enhanced cellular response *in vivo*, in terms of activation and cell numbers (10, 24). Although the function of ICOS on is most studied on T helper cells, the importance of ICOS on cytotoxic T cells is also starting to become clear. Antitumor responses mediated by $CD8^+$ T cells could be enhanced by ICOS (25, 26, 27). Moreover, expansion of $CD8^+$ T cells in an allograft rejection model was decreased after blocking ICOS (28, 29). In contrast, ICOS is not required for a prominent primary antiviral response (18, 19).

CTLA-4 (CD152), the inhibitory receptor that opposes CD28, also binds B7.1 and B7.2 and serves to downregulate T cell activation (30, 31). This is most clearly seen in mice that lack CTLA-4. These mice display severe T cell activation and expansion of the $CD4^+$ and $CD8^+$ T cell pool, which is considered to be driven by auto-antigens (32). The $CD4^+$ T cells present in these mice are differentiated, mostly into Th2 type $CD4^+$ T cells. The lymphoproliferative phenotype is dependent on signals generated by CD28, because abrogating CD28 and B7.1/B7.2 interaction by gene targeting or blocking agents completely abolishes this aberrant T cell activation (33-35).

Because CD28 and ICOS function similarly in many ways, we questioned which part of the elicited T cell activation and differentiation in CTLA-$4^{-/-}$ mice could be attributed to signals generated by ICOS. Therefore, the ICOS-B7RP-1 interaction was blocked using an antagonistic antibody (Ab) in mice deficient for CTLA-4. The

aberrant T cell activation and high T cell numbers normally observed in mice lacking CTLA-4 were reduced by abrogating ICOS signaling, indicating a role for ICOS in T cell expansion. This observation is supported by *in vitro* studies where ICOS can initiate T cell expansion by induction of cell cycle progression in the presence of exogenous IL-2. So, in the presence of a CD28 signal, ICOS contributes to expansion of primed T cells.

Materials & methods

Mice and treatment

Mice deficient for B7.1, B7.2 and CTLA-4 (CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$) (34) and CTLA-4$^{-/-}$ (32) on a 129/SvS4Jae background, were kindly provided by A. Sharpe (Brigham and Women's Hospital, Boston, MA). C57BL/6 wild type mice were purchased at Harlan. All mice were bred under specific pathogen free conditions, housed in accordance with institutional guidelines of American Association of Accreditation of Laboratory Animal Care (AALAC). In experiments neonatal CTLA-4$^{-/-}$ mice were used. These mice were treated from birth with 10 μg per gram body weight antagonistic αICOS Ab (clone 12A8) or 4 μg per gram body weight murine CTLA-4-Ig (kind gift from A. Coyle, Millennium Pharmaceuticals, Cambridge), which were injected i.p. on alternating days for a period of 14 days. CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice were used in experiments at 6-8 weeks of age and treated with 100 μg of agonistic αCD28 Ab PV-1, kindly provided by C. Broeren (University of Utrecht) (36). αCD28 Ab was injected i.v. on days 0, 7 and 14. Furthermore, these mice received 100 μg of either 12A8 or rat IgGs (own production) i.p. on alternating days, starting at day 0. Mice treated with CD4^{+} depleting Ab (GK1.5) received 50 μg i.p. on alternating days, starting 3 days before αCD28 treatment. Mice were sacrificed and lymph nodes were collected for analysis on day 17.

Flow cytometry

Cell suspensions were prepared from pooled isolated lymph nodes, counted and stained with a panel of fluorochrome-conjugated Abs, all purchased at BD Pharmingen, except for αICOS Ab (C398.4A, eBioscience). The Ab stainings were performed in PBS/0.5% BSA and 0.1% azide supplemented with 2% goat serum. Nonspecific FcR binding was prevented by preincubating cells on ice with FcR blocking Ab, clone 2.4G2 (BD Pharmingen) for 10 minutes. Subsequently, dilutions of the staining Abs were added and cells were incubated at 4°C for 30 minutes. CD4^{+} and CD8^{+} T cells were analyzed for CD25, CD69, CD62L, CD44, CD45RB and CD43 surface expression. Stained cells were washed twice before analysis on a flow cytometer (BD Biosciences).

T cell purification and T cell culture

CD4^{+} T cells or CD8^{+} T cells were purified from peripheral lymph nodes by positive selection with either αCD4 or αCD8 magnetic MACS beads (Miltenyi Biotec). Populations were reproducibly >98% pure. T cell populations were stimulated with magnetic beads (Dynal) covalently linked according to manufacturer's instructions with either αCD3 (145-2C11) hamster Ig (BD Pharmingen), αCD3-αCD28 (PV-1), or αCD3-αICOS (C398.4A, eBioscience) in 1:10 molar ratio. T cells were mixed in a 1:1 ratio with the Ab coated beads at 1x10^{5} cells per well and incubated at 37°C for several days.

Exogenous IL-2 was added at a concentration of 360 units/ml. After the indicated numbers of days supernatants were harvested for cytokine measurement.

Cell cycle analysis

Purified $CD4^+$ and $CD8^+$ T cells were labeled with the membrane dye carboxyfluorescein diacetate succinimidyl ester (CFSE) for analysis of T cell proliferation. $CD4^+$ and $CD8^+$ T cells were harvested after respectively 4 and 3 days of culture, respectively, counted and the reduction of signal of the CFSE dye per cell was analyzed using flow cytometry. Cell viability was assessed by resuspending cell pellets in Viaprobe dye (BD Pharmingen) followed by incubation at room temperature for 5 min before analysis. The total number of viable/non-viable cells after each round of division was calculated as follows. Cell populations that displayed similar dilution of the CFSE dye were gated, so that each gate represented cells that underwent an equal number of cell divisions. Within each gated population the percentage of Viaprobe negative and positive cells were distinguished. The absolute numbers of cells in the acquired gates were calculated by taking the respective percentage of the counted total T cell numbers. Cell debris and beads were excluded from the analysis.

ELISA

To detect cytokine production from *in vitro* stimulated $CD4^+$ T cells, supernatants were collected at the indicated time points. Cytokines were quantified by sandwich ELISA, purchased from BD Pharmingen (IL-2, IL-4, IL-10) or eBiosource (IFN-γ).

Statistical analysis

Results were analyzed using a Student's *t* test (two-tailed). Differences between groups were considered statistically significant at the $p<0.05$ level. Data is expressed as mean ± standard error of mean (SEM) unless indicated.

Results

ICOS expression is upregulated on $CD4^+$ and $CD8^+$ T cells by CD28 costimulation

First, the effect of CD28 costimulation on the augmentation of ICOS expression was tested *in vitro* by coculturing T cells with Ab coated beads. ICOS was readily detectable on the surface of both resting $CD4^+$ and $CD8^+$ T cells (**Fig. 1**). Stimulation with αCD3

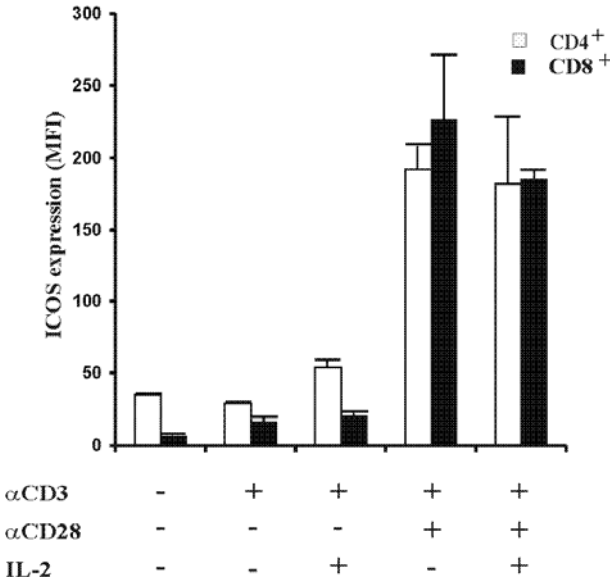

Figure 1. ICOS expression is enhanced by αCD28 stimulation. $CD4^+$ and $CD8^+$ T cells were purified from peripheral lymph nodes and were either directly stained with αICOS Ab or stained after stimulation with αCD3 or αCD3-αCD28 coated beads with or without exogenous IL-2 for 24 h. Expression levels of ICOS were analyzed by flow cytometry. Indicated are means of duplicates ± SD. Shown is a representative of two independent experiments.

in the presence of exogenous IL-2 enhanced the expression of ICOS to some degree on $CD4^+$ T cells, whereas on $CD8^+$ T cells IL-2 did not elevate the level of ICOS compared with αCD3 alone. Additional stimulation with αCD28 greatly enhanced the expression levels of ICOS on both $CD4^+$ and $CD8^+$ T cells, independent of the addition of IL-2. So, expression of ICOS can be upregulated by signals generated by CD28 in conjunction with TCR signaling. These results prompted us to study the role of ICOS in CTLA-4 deficient mice, where a TCR driven immune response was enhanced by a strong CD28 signal.

Blocking ICOS inhibits expansion of T cells in mice lacking CTLA-4

The contribution of ICOS to an *in vivo* immune response in the presence of a CD28 signal was determined in mice lacking CTLA-4. As expected, T cells from these mice had a 5- to 6-fold enhanced expression of ICOS when analyzed *ex vivo* (data not shown). Mice lacking CTLA-4 were treated from birth with the antagonistic αICOS Ab 12A8. As previously reported, this Ab functionally blocks the interaction between ICOS and its natural ligand B7RP-1 *in vivo* (21, 29, 37). Interestingly, administration of blocking αICOS Ab caused a 4-fold reduction in the normally observed expanded T cell pool in peripheral lymph nodes of CTLA-$4^{-/-}$ mice (**Fig. 2**). As shown previously, treatment with CTLA-4-Ig, preventing CD28 and B7.1/B7.2 interaction, completely reduced T cell numbers back to levels found in wild type animals (33). Treatment with either CTLA-4-Ig or blocking αICOS Ab alone did not affect T cell numbers in wild type mice (data not shown). So, the expansion of the T cell pool in mice lacking CTLA-4 is partly dependent on signals generated by ICOS-B7RP-1 interaction.

ICOS and CD28 both contribute to $CD4^+$ and $CD8^+$ T cells expansion in the absence of CTLA-4

To look in more detail at the contribution of ICOS to CD28 mediated T cell expansion *in vivo*, we used mice lacking CTLA-4 and B7.1/2 (CTLA-$4^{-/-}$ B7.1/B7.$2^{-/-}$).

The advantage of using this strain over mice solely deficient for CTLA-4 is that in adult CTLA-$4^{-/-}$ B7.1/B7.$2^{-/-}$ mice a strong CD28 signal can be generated by

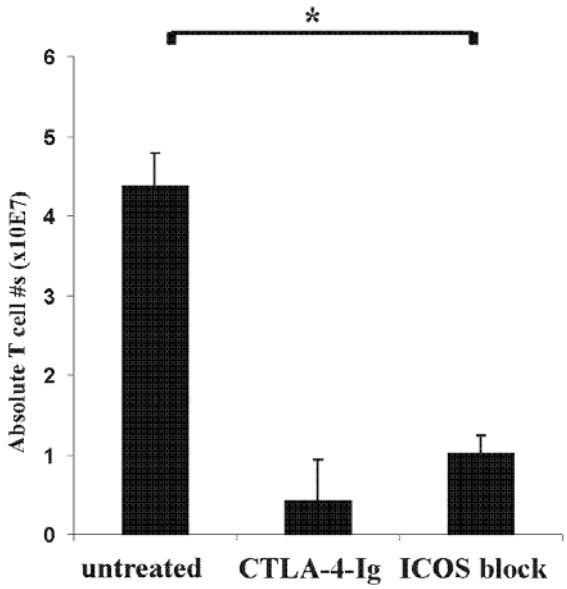

Figure 2. Blocking ICOS partly prevents lymphoproliferation in CTLA-$4^{-/-}$ mice. Mice deficient for CTLA-4 were treated from birth with CTLA-4-Ig or blocking αICOS Ab by injection i.p. on alternating days and were sacrificed on day 14. Peripheral lymph nodes were isolated and single cell suspensions were prepared. The percentage of $CD3^+$ T cells was analyzed by cell surface staining and flow cytometry. Absolute numbers were determined by calculating the percentages of $CD3^+$ cells of the total number of cells isolated from peripheral lymph nodes. Indicated are means ± SEM (* = $p<0.05$). Three animals were analyzed per treatment.

administration of agonistic αCD28 Ab. This way any disorders by having CD28 signals present in the absence of CTLA-4 during development can be circumvented and the T cell responses can be studied in adult mice. As shown previously, injection of agonistic αCD28 Ab in CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice causes vigorous T cell expansion, reflected in an increase in CD69 expression as a marker for T cell activation and a 10- and 20-fold increase in CD8^{+} and CD4^{+} T cell numbers respectively in peripheral lymph nodes (34) (**Fig. 3A and 3B**). The peripheral T cell pool in these treated mice was also affected by antagonistic αICOS Ab treatment, like that observed in the CTLA-4 $^{-/-}$ mice, in terms of T cell numbers and T cell activation. Both CD4^{+} and CD8^{+} T cell numbers in the lymph nodes were 2-fold reduced when stimulating αCD28 Ab was coinjected with blocking αICOS Ab, compared with αCD28 Ab alone (**Fig. 3A**). But, T cell numbers of mice that received both agonistic αCD28 Ab and antagonistic αICOS Ab were still higher than T cell numbers of mice that did not receive treatment, indicating that CD28 mediated CD4^{+} and CD8^{+} T cell expansion was partially independent of ICOS. T cells from mice treated with blocking αICOS Ab alone remained naïve and were similar in absolute numbers compared with untreated animals (data not shown).

To strengthen the observation that ICOS plays a role in T cell expansion, we looked at the activation state of CD4^{+} and CD8^{+} T cells phenotypically. The percentage of CD69 expressing CD4^{+} as well as CD8^{+} T cells was decreased when ICOS was blocked (**Fig. 3B**, *top and bottom, panel 3*), but did not decline to expression levels found on T cells from untreated mice (**Fig. 3B**, *top and bottom, panel 1*). The lower activation status following ICOS blockade was confirmed by analysis of other markers of activation, like upregulation of CD25 and downregulation of CD62L (data not shown). So, blocking ICOS in αCD28-treated CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice results in reduced activation and expansion of CD4^{+} and CD8^{+} T cells.

Previously it was shown that CD8^{+} T cell expansion in CTLA-4$^{-/-}$ mice is dependent on the presence of CD4^{+} T cells (38). We therefore questioned whether the effect of ICOS blockade on CD8^{+} T cell numbers was caused by direct inhibition of CD8^{+} T cell proliferation or indirectly due to blocking of CD4^{+} T cell activation and expansion in our model. First, we investigated CD8^{+} T cell expansion in CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ after αCD28 treatment in the presence of CD4^{+} depletion Abs. Absence of CD4^{+} T cells in αCD28-treated CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice resulted in complete normalization of the CD8^{+} T cell pool, leaving CD8^{+} T cell numbers similar to untreated animals (**Fig. 3C**). These results show that for CD8^{+} T cells to proliferate the presence of CD4^{+} T cells is required. However, this excludes the possibility of testing the direct effect of ICOS on CD8^{+} T cell expansion in αCD28-treated CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice. Taken together, ICOS contributes directly to CD28-mediated CD4^{+} T cell activation and expansion in the absence of CTLA-4.

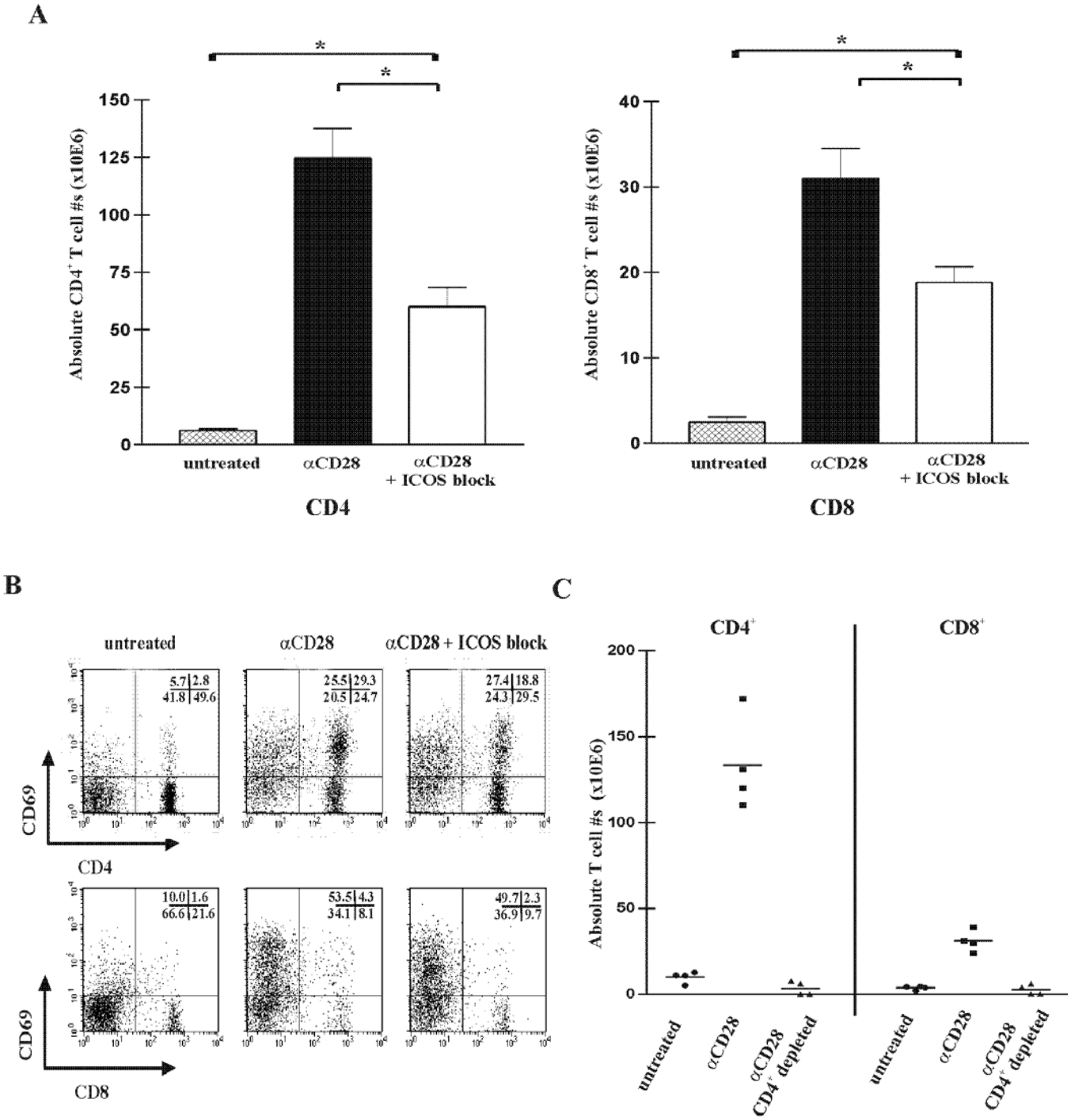

Figure 3. Blocking ICOS inhibits expansion of CD4+ and CD8+ T cells in mice deficient for CTLA-4/B7.1/B7.2. CTLA-4-/- B7.1/B7.2-/- mice were injected i.v. with agonistic αCD28 Ab (PV-1) on day 0, 7 and 14, with additional antagonistic αICOS Ab or isotype control Ab i.p. on alternating days, starting on day 0. Mice were sacrificed on day 17 and cell suspensions were prepared from pooled peripheral lymph nodes. **A.** Absolute numbers of CD4+ and CD8+ T cells in peripheral lymph nodes. Cells were stained with αCD4 and αCD8 Ab and the percentages of each subset were determined by flow cytometry. Absolute numbers of CD4+ and CD8+ T cells were calculated by the percentage of positive cells of the total number of lymph node cells. Values represent means ± SEM (* = $p<0.05$). **B.** Comparison of the expression of activation markers on CD4+ and CD8+ T cells. Cells were stained with αCD4, αCD8 and αCD69 Ab and analyzed by flow cytometry. Percentages of cells in each quadrant are indicated. FACS plots show a representative of three independent experiments. A minimum of seven mice was analyzed for each treatment. **C.** Absolute T cell numbers after CD4+ T cell depletion. In addition to αCD28 treatment, CTLA-4-/- B7.1/B7.2-/- mice received CD4+ depletion Ab on alternating days, starting at day -3. Mice were sacrificed on day 17 and absolute T cell numbers were calculated as in **A**. Four mice were analyzed per group.

ICOS partly drives Th1 and Th2 differentiation in mice lacking CTLA-4

Next, the contribution of ICOS to CD28 induced $CD4^+$ Th1 and Th2 cell differentiation was assessed in CTLA-$4^{-/-}$ B7.1/B7.$2^{-/-}$ mice. Cytokines produced by $CD4^+$ T cells from these mice injected with αCD28 Ab in the absence or presence of blocking αICOS Ab were determined *in vitro*. Treatment of CTLA-$4^{-/-}$ B7.1/B7.$2^{-/-}$ mice with agonistic αCD28 Ab caused $CD4^+$ T cell differentiation towards Th1 and Th2 type cells, producing IFNγ and IL-4/10, respectively (**Fig. 4**). When treated simultaneously with antagonistic αICOS Ab there was a noticeable (but not statistically different) decrease in IL-4 and IL-10 cytokine production. Although the Th1 cytokine levels were much lower compared with the Th2 cytokine levels, there was a significant reduction in IFNγ production after blocking ICOS. IL-2 production was similar between the two groups. $CD4^+$ T cells from untreated CTLA-$4^{-/-}$ B7.1/B7.$2^{-/-}$ mice failed to produce any cytokines after *in vitro* stimulation (data not shown). Overall, there is a reduction in cytokine production from both Th1 and Th2 type T cells, indicating that cells from mice treated with blocking αICOS Ab were less differentiated. Thus, ICOS is involved in both Th1 and Th2 differentiation driven by CD28 signaling.

ICOS induced division of $CD4^+$ and $CD8^+$ T cells is dependent on IL-2 in vitro

The observed decrease in expansion of the $CD4^+$ T cell pool after abrogation of ICOS function could either be due to less cell division or increased cell death. To distinguish between these two putative consequences of ICOS blocking, the contribution of ICOS to both cell cycle progression and cell survival was further investigated *in vitro*. Purified wild type $CD4^+$ T cells were labeled with CFSE to be able to track numbers of cells that go through cell cycle by flow cytometry. Viability of cells going through rounds of division was analyzed simultaneously by using Viaprobe as a fluorescent dye, which only enters the cell when the membrane

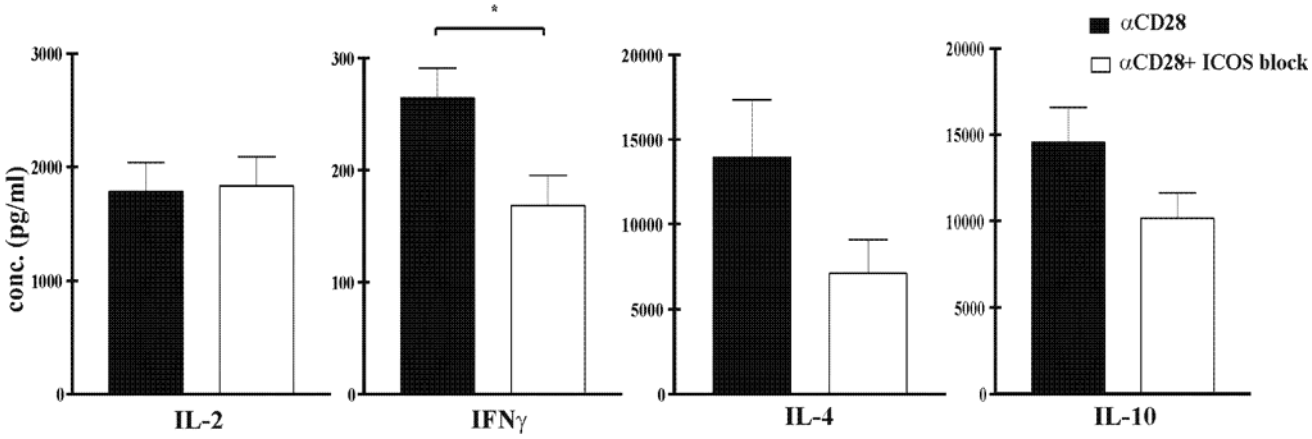

Figure 4. ICOS and CD28 both contribute to Th1 and Th2 development. Purified $CD4^+$ T cells were isolated from peripheral lymph nodes of CTLA-$4^{-/-}$ B7.1/B7.$2^{-/-}$ treated with αCD28 with or without blocking αICOS Ab. Similar numbers of $CD4^+$ T cells were stimulated in vitro with αCD3-αCD28 coated beads for a period of three days. Supernatants were collected and cytokines were measured by sandwich ELISA. Indicated are IL-2 levels on day 1, IFN-γ on day 2 and IL-4/10 on day 3. A minimum of seven mice was analyzed for each treatment. Values represent means ± SEM (* = $p<0.05$).

integrity is compromised. Results are shown in **fig. 5A**, where the total numbers of viable and nonviable $CD4^+$ T cells that have undergone the indicated number of cell divisions are displayed. Most of the T cells stimulated with αCD3 alone underwent only one or two cell divisions (**Fig. 5A**, *panel 1*). However, after simultaneous co-crosslinking with CD28 (**Fig. 5A**, *middle panel*) cells went through four to six rounds of division. In both of these stimulation conditions exogenous IL-2 did not significantly influence cell division. In contrast, addition of IL-2 considerably changed the outcome of the response after αCD3-αICOS stimulation. As depicted in the third top/bottom panels (**Fig. 5A**), cell cycle progression of $CD4^+$ T cells could only be induced by ICOS in the presence of IL-2 (**Fig. 5A**). Because the majority of the cells progressed through cell cycle, it is difficult to draw conclusions about an effect on cell survival by ICOS. Still, the total amount of non-viable αCD3-αICOS stimulated cells that have gone through one cell division was unchanged compared with T cells that were stimulated by αCD3 alone, indicating that ICOS may not promote cell survival in cells that were refractory to further cell division (**Fig. 5A**, *left and right lower panels*, black bars).

To address the question whether there can be a direct effect of ICOS on $CD8^+$ T cell expansion, we performed similar proliferation assays using purified wild type $CD8^+$ T cells. As shown in **fig. 5B**, $CD8^+$ T cells are able to progress through cell cycle in response to αCD3-αICOS stimulation, but only in the presence of exogenous IL-2. Taken together, ICOS induced cell cycle progression of both $CD4^+$ and $CD8^+$ T cells in the presence of IL-2. Moreover, ICOS did not rescue cells that had not progressed through cell cycle from cell death.

Discussion

We here report a study in which the contribution of ICOS to an *in vivo* immune response was investigated in CTLA-4 deficient mice. When ICOS was blocked in $CTLA\text{-}4^{-/-}$ mice, the aberrant activation of $CD4^+$ T cells and expansion of both $CD4^+$ and $CD8^+$ T cells was reduced. Moreover, CD28 mediated differentiation towards Th1 and Th2 type $CD4^+$ T cells was partly dependent on ICOS. Further in *vitro* studies showed that ICOS induced cell cycle progression of both $CD4^+$ and $CD8^+$ T cells, which was dependent on exogenous IL-2. Taken together, in the presence of CD28 signaling, ICOS functions early in the cellular response, contributing to expansion of T cells and differentiation of $CD4^+$ T cells.

The results presented here shed new light on the role of ICOS in the expansion phase of a T cell response. These data are in accordance with other studies, which also show that ICOS enhances T cell activation and expansion both *in vitro* and *in vivo* (11, 24, 37). In contrast, several blocking studies during different phases of an immune response showed the most pronounced effect when ICOS signaling is abrogated later in the immune response (21, 28). It is possible that a delicate balance between TCR signal and CD28 determines the requirement for ICOS early in the response. When comparing the contribution of ICOS and CD28 to the initial phase of the immune response, we hypothesize that CD28 is the most important molecule for initial activation and expansion of T cells.

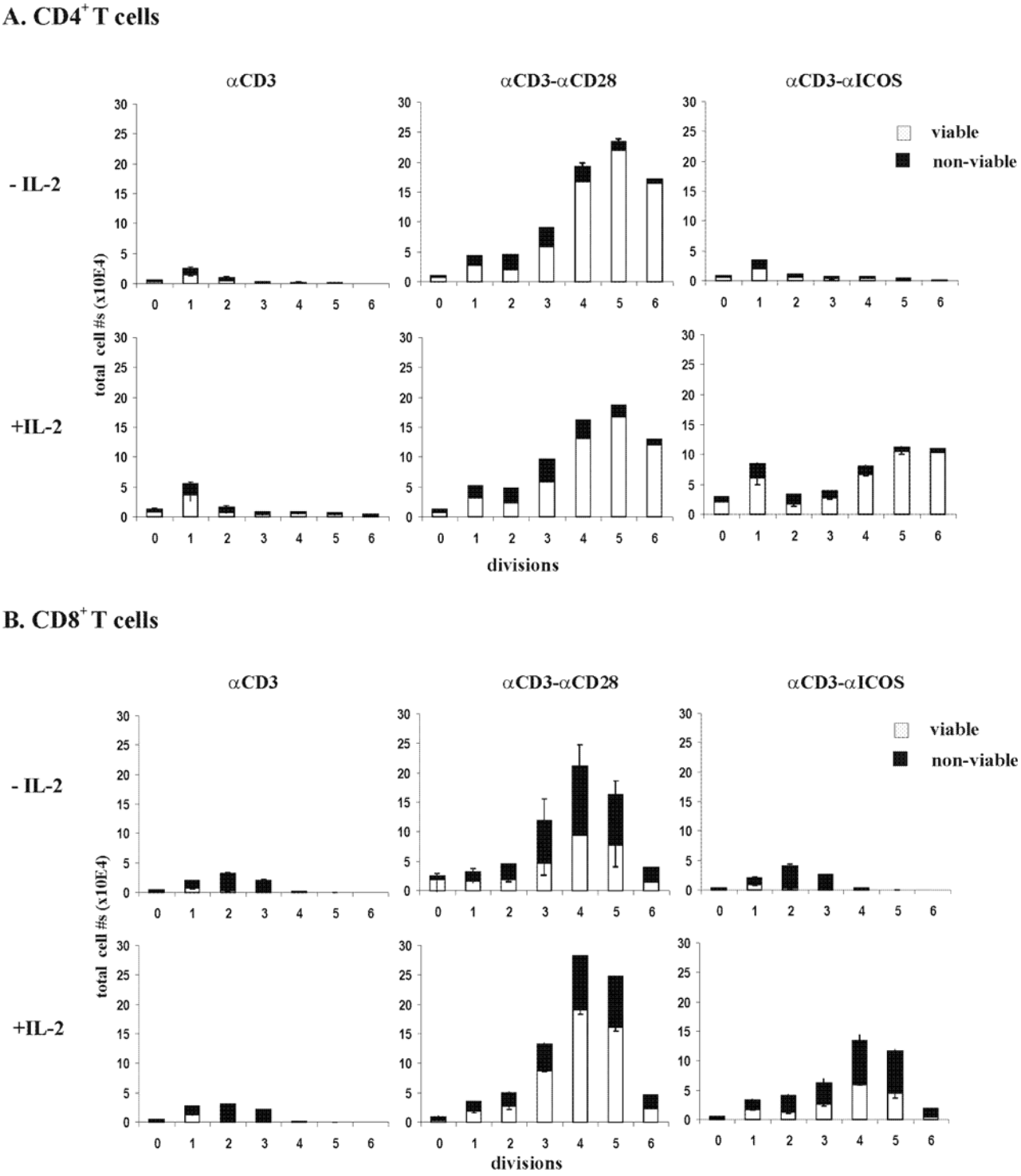

Figure 5. ICOS induces cell cycle progression of CD4+ **(A)** and CD8+ **(B)** T cells in the presence of IL-2. Peripheral lymph node T cells were purified from wild type mice, labelled with CFSE and activated with Ab coated beads as indicated, with or without addition of exogenous IL-2. CD8+ T cells were harvested after three days and CD4+ T cells after four days. The percentage of cells that went through the indicated number of divisions was determined by dilution of the CFSE label, while cell viability was simultaneously analyzed with Viaprobe as a dye for non-viable cells. Cultured cells were counted and total viable (white bars) and non-viable (black bars) T cell numbers, which had undergone the indicated number of cell divisions, were calculated using the percentages of each population of the total number of T cells. Indicated are means of duplicates ± SD.

This can be explained not only by low expression of ICOS on naïve T cells, but might also lie in the fact that ICOS, unlike CD28, cannot induce IL-2 production in murine T cells (11, 22). The importance of CD28 as initiator molecule is also seen in CTLA-4$^{-/-}$ mice, where blocking of CD28 completely abolishes T cell activation, whereas ICOS blocking only partly does so. Still, we have shown that CD28 does not induce a complete T cell response in the absence of ICOS in αCD28-treated CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice. The contribution of ICOS to T cell expansion might be more pronounced when T cell activation occurs after signals generated by CD28, as suggested by the strong ICOS upregulation after TCR-CD28 coligation and the necessity of IL-2 for ICOS induced proliferation *in vitro*. In conclusion, in the absence of CTLA-4, although CD28 functions as an initiator molecule, sequential engagement of ICOS has an additive effect on T cell expansion.

The observed reduction in peripheral T cells could result from less division or increased cell death. We conclude from our *in vitro* T cell stimulation assay that ICOS, in the presence of IL-2, is able to induce cell cycle progression. Therefore, it is likely that the observed inhibition of T cell expansion after *in vivo* blockade of B7RP-1-ICOS interaction in CTLA-4$^{-/-}$ is caused by reduced cell proliferation. We cannot exclude that abrogation of ICOS signaling could have caused increased cell death *in vivo.* Whether ICOS induces an intrinsic cell survival mechanism independent from proliferation, like CD28 does by up-regulation of Bcl-x$_L$ (39, 40) is currently under investigation.

One way to explain the results obtained here is by using the term "cell fitness", first proposed by Lanzavecchia and Sallusto (41). When optimal signals from the TCR, costimulatory molecules and cytokine receptors are integrated, cells go through cell division acquiring resistance to apoptosis and susceptibility to cytokines like IL-2, IL-15 and IL-7. In the present study cells stimulated with αCD3 alone or αCD3-αICOS cells can be termed as "unfit" cells. The signals provided are suboptimal; therefore no cell cycle progression occurs. When IL-2 is provided in the presence of a TCR signal and ICOS, these cells obtain a higher level of "fitness" and proceed through cell cycle remaining viable. The importance of IL-2 is also supported by studies in human CD4$^+$ T cells, where neutralizing IL-2 Ab can inhibit ICOS induced proliferation (42). We and others (43) have observed that IL-2 increases ICOS expression on murine T cells *in vitro.* However, it is unclear whether this upregulation is mandatory for the induction of proliferation via ICOS. Other possible mechanisms by which IL-2 can enhance ICOS induced proliferation in TCR triggered cells remain to be determined.

Next to the effect on CD4$^+$ T cell expansion, we have found a clear effect of ICOS on CD8$^+$ T cell expansion in the absence of CTLA-4. In agreement with these results, ligation of ICOS on CD8$^+$ T cells induced proliferation in the presence of IL-2 *in vitro*. However, depletion of CD4$^+$ T cells in αCD28-treated CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice showed that the expansion of the CD8$^+$ T cells is dependent on the presence of CD4$^+$ T cells. Therefore, we cannot conclude from our model that ICOS has a direct effect on CD8$^+$ T cell expansion *in vivo*.

The assumption that therapeutic interventions in the ICOS/B7RP-1 pathway

is restricted to the effector phase may have to be revised, because our data show that ICOS plays a role in the expansion phase of a T cell response. CD28 is believed to be one of the most important initiator molecules of an immune response discovered to date; so blocking CD28 could have major implications when used in a therapeutical setting. Future therapies targeting ICOS rather than CD28 may have to take into account that abrogation of ICOS signaling may not only affect secondary responses, but may also inhibit earlier in cellular responses.

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References

1. Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu.Rev.Immunol.* 14:233-58.:233-258.
2. Sharpe, A. H. and G. J. Freeman. 2002. The B7-CD28 superfamily. *Nat.Rev. Immunol.* 2:116-126.
3. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261:609-612.
4. Green, J. M., P. J. Noel, A. I. Sperling, T. L. Walunas, G. S. Gray, J. A. Bluestone, and C. B. Thompson. 1994. Absence of B7-dependent responses in CD28-deficient mice. *Immunity* 1:501-508.
5. Noel, P. J., L. H. Boise, J. M. Green, and C. B. Thompson. 1996. CD28 costimulation prevents cell death during primary T cell activation. *J.Immunol.* 157:636-642.
6. Salomon, B. and J. A. Bluestone. 2001. Complexities of CD28/B7: CTLA-4 costimulatory pathways in auto-immunity and transplantation. *Annu. Rev.Immunol.* 19:225-52.:225-252.
7. Hendriks, J., Y. Xiao, and J. Borst. 2003. CD27 promotes survival of activated T cells and complements CD28 in generation and establishment of the effector T cell pool. *J.Exp.Med.* 198:1369-1380.
8. Andreasen, S. O., J. E. Christensen, O. Marker, and A. R. Thomsen. 2000. Role of CD40 ligand and CD28 in induction and maintenance of antiviral $CD8^+$ effector T cell responses. *J.Immunol.* 164:3689-3697.
9. Bertram, E. M., W. Dawicki, and T. H. Watts. 2004. Role of T cell costimulation in anti-viral immunity. *Semin.Immunol.* 16:185-196.
10. Yoshinaga, S. K., J. S. Whoriskey, S. D. Khare, U. Sarmiento, J. Guo, T. Horan, G. Shih, M. Zhang, M. A. Coccia, T. Kohno, A. Tafuri-Bladt, D. Brankow, P. Campbell, D. Chang, L. Chiu, T. Dai, G. Duncan, G. S. Elliott, A. Hui, S. M. McCabe, S. Scully, A. Shahinian, C. L. Shaklee, G. Van, T. W. Mak, and . 1999. T-cell co-stimulation through B7RP-1 and ICOS. *Nature* 402:827-832.
11. Hutloff, A., A. M. Dittrich, K. C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos, and R. A. Kroczek. 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 397:263-266.
12. Tafuri, A., A. Shahinian, F. Bladt, S. K. Yoshinaga, M. Jordana, A. Wakeham, L. M. Boucher, D. Bouchard, V. S. Chan, G. Duncan, B. Odermatt, A. Ho, A. Itie, T. Horan, J. S. Whoriskey, T. Pawson, J. M. Penninger, P. S. Ohashi, and T. W. Mak. 2001. ICOS is essential for effective T-helper-

cell responses. *Nature* 409:105-109.

13. McAdam, A. J., R. J. Greenwald, M. A. Levin, T. Chernova, N. Malenkovich, V. Ling, G. J. Freeman, and A. H. Sharpe. 2001. ICOS is critical for CD40-mediated antibody class switching. *Nature* 409:102-105.
14. Dong, C., A. E. Juedes, U. A. Temann, S. Shresta, J. P. Allison, N. H. Ruddle, and R. A. Flavell. 2001. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 409:97-101.
15. Suh, W. K., A. Tafuri, N. N. Berg-Brown, A. Shahinian, S. Plyte, G. S. Duncan, H. Okada, A. Wakeham, B. Odermatt, P. S. Ohashi, and T. W. Mak. 2004. The inducible costimulator plays the major costimulatory role in humoral immune responses in the absence of CD28. *J.Immunol.* 172:5917-5923.
16. Coyle, A. J., S. Lehar, C. Lloyd, J. Tian, T. Delaney, S. Manning, T. Nguyen, T. Burwell, H. Schneider, J. A. Gonzalo, M. Gosselin, L. R. Owen, C. E. Rudd, and J. C. Gutierrez-Ramos. 2000. The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. *Immunity* 13:95-105.
17. Greenwald, R. J., A. J. McAdam, W. D. Van der, A. R. Satoskar, and A. H. Sharpe. 2002. Cutting edge: inducible costimulator protein regulates both Th1 and Th2 responses to cutaneous leishmaniasis. *J.Immunol.* 168:991-995.
18. Bertram, E. M., A. Tafuri, A. Shahinian, V. S. Chan, L. Hunziker, M. Recher, P. S. Ohashi, T. W. Mak, and T. H. Watts. 2002. Role of ICOS versus CD28 in antiviral immunity. *Eur.J.Immunol.* 32:3376-3385.
19. Kopf, M., A. J. Coyle, N. Schmitz, M. Barner, A. Oxenius, A. Gallimore, J. C. Gutierrez-Ramos, and M. F. Bachmann. 2000. Inducible costimulator protein (ICOS) controls T helper cell subset polarization after virus and parasite infection. *J.Exp.Med.* 192:53-61.
20. Miyahira, Y., H. Akiba, S. H. Ogawa, T. Ishi, S. Watanabe, S. Kobayashi, T. Takeuchi, T. Aoki, K. Tezuka, R. Abe, K. Okumura, H. Yagita, and N. Watanabe. 2003. Involvement of ICOS-B7RP-1 costimulatory pathway in the regulation of immune responses to *Leishmania major* and *Nippostrongylus brasiliensis* infections. *Immunol.Lett.* 89:193-199.
21. Gonzalo, J. A., J. Tian, T. Delaney, J. Corcoran, J. B. Rottman, J. Lora, A. Al garawi, R. Kroczek, J. C. Gutierrez-Ramos, and A. J. Coyle. 2001. ICOS is critical for T helper cell-mediated lung mucosal inflammatory responses. *Nat.Immunol.* 2:597-604.
22. McAdam, A. J., T. T. Chang, A. E. Lumelsky, E. A. Greenfield, V. A. Boussiotis, J. S. Duke-Cohan, T. Chernova, N. Malenkovich, C. Jabs, V. K. Kuchroo, V. Ling, M. Collins, A. H. Sharpe, and G. J. Freeman. 2000. Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of $CD4^+$ T cells. *J.Immunol.* 165:5035-5040.
23. Nurieva, R. I., X. M. Mai, K. Forbush, M. J. Bevan, and C. Dong. 2003. B7h is required for T cell activation, differentiation, and effector function. *Proc.Natl.Acad.Sci.U.S.A.* 100:14163-14168.
24. Guo, J., M. Stolina, J. V. Bready, S. Yin, T. Horan, S. K. Yoshinaga, and G. Senaldi. 2001. Stimulatory effects of B7-related protein-1 on cellular and humoral immune responses in mice. *J.Immunol.* 166:5578-5584.
25. Ara, G., A. Baher, N. Storm, T. Horan, C. Baikalov, E. Brisan, R. Camacho, A. Moore, H. Goldman, T. Kohno, R. C. Cattley, G. Van, K. Gaida, M. Zhang, J. S. Whoriskey, D. Fong, and S. K. Yoshinaga. 2003. Potent activity of soluble B7RP-1-Fc in therapy of murine tumors in syngeneic hosts. *Int.J.Cancer* 103:501-507.
26. Wallin, J. J., L. Liang, A. Bakardjiev, and W. C. Sha. 2001. Enhancement of $CD8^+$ T cell responses by ICOS/B7h costimulation. *J.Immunol.* 167:132-139.
27. Liu, X., X. F. Bai, J. Wen, J. X. Gao, J. Liu, P.

Lu, Y. Wang, P. Zheng, and Y. Liu. 2001. B7H costimulates clonal expansion of, and cognate destruction of tumor cells by, CD8(+) T lymphocytes *in vivo*. *J.Exp.Med.* 194:1339-1348.

28. Harada, H., A. D. Salama, M. Sho, A. Izawa, S. E. Sandner, T. Ito, H. Akiba, H. Yagita, A. H. Sharpe, G. J. Freeman, and M. H. Sayegh. 2003. The role of the ICOS-B7h T cell costimulatory pathway in transplantation immunity. *J.Clin.Invest.*112:234-243.
29. Ozkaynak, E., W. Gao, N. Shemmeri, C. Wang, J. C. Gutierrez-Ramos, J. Amaral, S. Qin, J. B. Rottman, A. J. Coyle, and W. W. Hancock. 2001. Importance of ICOS-B7RP-1 costimulation in acute and chronic allograft rejection. *Nat.Immunol.* 2:591-596.
30. Thompson, C. B. and J. P. Allison. 1997. The emerging role of CTLA-4 as an immune attenuator. *Immunity* 7:445-450.
31. Oosterwegel, M. A., R. J. Greenwald, D. A. Mandelbrot, R. B. Lorsbach, and A. H. Sharpe. 1999. CTLA-4 and T cell activation. *Curr.Opin.Immunol.* 11:294-300.
32. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541-547.
33. Tivol, E. A., S. D. Boyd, S. McKeon, F. Borriello, P. Nickerson, T. B. Strom, and A. H. Sharpe. 1997. CTLA4Ig prevents lymphoproliferation and fatal multi-organ tissue destruction in CTLA-4-deficient mice. *J.Immunol.* 158:5091-5094.
34. Mandelbrot, D. A., A. J. McAdam, and A. H. Sharpe. 1999. B7-1 or B7-2 is required to produce the lympho-proliferative phenotype in mice lacking cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). *J.Exp.Med.* 189:435-440.
35. Mandelbrot, D. A., M. A. Oosterwegel, K. Shimizu, A. Yamada, G. J. Freeman, R. N. Mitchell, M. H. Sayegh, and A. H. Sharpe. 2001. B7-dependent T-cell costimulation in mice lacking CD28 and CTLA4. *J.Clin.Invest.*107:881-887.
36. Abe, R., P. Vandenberghe, N. Craighead, D. S. Smoot, K. P. Lee, and C. H. June. 1995. Distinct signal transduction in mouse $CD4^+$ and $CD8^+$ splenic T cells after CD28 receptor ligation. *J.Immunol.* 154:985-997.
37. Smith, K. M., J. M. Brewer, P. Webb, A. J. Coyle, C. Gutierrez-Ramos, and P. Garside. 2003. Inducible costimulatory molecule-B7-related protein 1 interactions are important for the clonal expansion and B cell helper functions of naïve, Th1, and Th2 T cells. *J.Immunol.* 170:2310-2315.
38. Chambers, C. A., T. J. Sullivan, and J. P. Allison. 1997. Lymphoproliferation in CTLA-4-deficient mice is mediated by costimulation-dependent activation of $CD4^+$ T cells. *Immunity* 7:885-895.
39. Boise, L. H., A. J. Minn, P. J. Noel, C. H. June, M. A. Accavitti, T. Lindsten, and C. B. Thompson. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity* 3:87-98.
40. Okkenhaug, K., L. Wu, K. M. Garza, J. La Rose, W. Khoo, B. Odermatt, T. W. Mak, P. S. Ohashi, and R. Rottapel. 2001. A point mutation in CD28 distinguishes proliferative signals from survival signals. *Nat.Immunol.* 2:325-332.
41. Lanzavecchia, A. and F. Sallusto. 2002. Progressive differentiation and selection of the fittest in the immune response. *Nat.Rev.Immunol.* 2:982-987.
42. Riley, J. L., P. J. Blair, J. T. Musser, R. Abe, K. Tezuka, T. Tsuji, and C. H. June. 2001. ICOS costimulation requires IL-2 and can be prevented by CTLA-4 engagement. *J.Immunol.* 166:4943-4948.
43. Yagi, J., Y. Arimura, U. Dianzani, T. Uede, T. Okamoto, and T. Uchiyama. 2003. Regulatory roles of IL-2 and IL-4 in H4/inducible costimulator expression on activated $CD4^+$ T cells during Th cell development. *J.Immunol.* 171:783-794.

Chapter 4

Hax-1 associates with ICOS and CD28 in yeast and is required for CD28-induced IL-2 production in human T cells

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Summary
CD28 and ICOS (Inducible Costimulator) are two homologues that belong to the B7-receptor family of costimulatory molecules. Both function in addition to the Ag-specific signal generated by the TCR, leading to T cell proliferation and differentiation. Moreover, CD28 delivers a cell survival signal mediated through pro-survival protein Bcl-X_L. Since CD28 and ICOS are similar, we questioned whether CD28 and ICOS induce similar pro-survival genes in human $CD4^+$ T cells. Like CD28, ICOS-mediated costimulation increased induction of Bcl-X_L protein levels. In addition, we searched for new intracellular binding partners that could mediate costimulation-specific functions. Hax-1 was identified as an ICOS and CD28 associating molecule in yeast and was found to mediate CD28-induced IL-2 production in primary human $CD4^+$ T cells.

Introduction

CD28 is an important costimulatory molecule which, in conjunction with the TCR, serves as the major activator of naïve T cells. Interaction between CD28 and its ligands B7.1 (CD86) and/or B7.2 (CD86) results in clonal expansion, IL-2 production and T cell survival that is mediated by the induction of pro-survival protein Bcl-X_L (1-3). CD28 is expressed as a homodimer of 45 kDa, with an intracellular tail containing two proline rich regions and four tyrosine residues (Y), one of which is embedded into a highly conserved YxxM motif (4). The identification of several intracellular binding partners has contributed to the understanding of downstream pathways that mediate CD28-induced T cell activation. The p85 subunit of PI3K associates with the phosphorylated tyrosine residue within the YMNM motif of the CD28 intracellular tail (5). Mutation of this tyrosine residue abrogates Bcl-X_L upregulation and resistance to cell death, suggesting a role for PI3K in CD28-mediated T cell survival (6, 7). The asparagine residue (N) within this motif is mandatory for association of Grb-2 and mutation of this AA reduced IL-2 promoter activity, suggesting a role for Grb-2 in CD28-induced IL-2 production (8-10).

Although some reports describe the importance of both tyrosine and asparagine residues for CD28-induced proliferation, this remains controversial (11). The inhibitory CD28 counterpart, CTLA-4, functions to negatively regulate T cell activation (12, 3). The intracellular tail of CTLA-4 is 100% conserved between man and mouse and contains docking site for several molecules, some of which are shared with CD28, like Pp2a and PI3K (4).

ICOS (Inducible Costimulator) has been identified as a CD28 homologue and associates with its specific ligand B7RP-1 (14, 5). Next to structural resemblance, also functional overlap has been described for the two molecules, such as induction of proliferation and cytokine production. The most prominent difference between ICOS and CD28 is the ability to induce IL-2: while CD28-mediated costimulation can induce large quantities of IL-2, ICOS is less capable of doing so. Also, both molecules have differential expression pattern: whereas ICOS expression is low on naïve cells and is upregulated after activation, CD28 is constitutively expressed on naïve T cells.

Despite well-studied functions of ICOS, signaling pathways preceding ICOS-mediated gene induction are ill defined. The ICOS intracellular tail is shorter in comparison to CD28 (21 AA vs. 41 AA, respectively) but shares the common YxxM motif. Similar to CD28, p85 subunit of PI3K associates to the motif YMFM in the ICOS tail (16). Moreover, TCR-induced PI3K activation was more enhanced following ICOS- compared to CD28-mediated costimulation (17). The difference between asparagine and a phenylalanine residue at position three within this motif in CD28 and ICOS tail respectively has been implicated to account for the difference IL-2 production, due to the inability of ICOS to recruit Grb-2 (18). So far no associating protein has been identified in ICOS-mediated proliferation and cytokine production.

In this study we investigated whether ICOS functions similarly to CD28 in the induction of cell survival in primary human $CD4^+$ T cells. In addition, we searched for new intracellular binding partners for CD28

and ICOS. Using a yeast-2-hybrid we demonstrate that Hax-1 associates with both CD28 and ICOS. We provide evidence that Hax-1 can be involved in CD28-mediated IL-2 production of human CD4+ T cells.

Materials & Methods

CD4+ T cell purification and CD4+ T cell culture

Human CD4+ T cells were purified from peripheral blood by Ficoll-Paque density gradient centrifugation (Ficoll-Paque plus™, Amersham Biosciences) followed by positive selection with αCD4 magnetic MACS beads (Miltenyi Biotec). Populations were reproducibly >98% pure as determined by flow cytometry. All cells were cultured in RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, UK), supplemented with 10% heat-inactivated fetal calf serum (FCS) (Fetalclone I, Hyclone, Logan, UT), 50 IU/ml penicillin (Gibco BRL) and 50 μg/ml streptomycin (Gibco BRL). CD4+ T cells were stimulated with αCD3 (OKT3)-hamsterIgG (Pharmingen), αCD3-αCD28 (15E8), or αCD3-αICOS (C398.4A, eBioscience) covalently linked to magnetic beads (Dynal) according to manufacture's instructions. Beads were coated with a suboptimal amount of αCD3 (7.5% of total protein) and either αICOS (40% of total protein) or αCD28 (αCD28: 5% of total protein and $\alpha CD28^{hi}$: 40% of total protein) supplemented with murine IgG1 to final amount of 5 μg/10^7 beads. T cells were mixed in a 1:1 ratio with the Ab-coated beads at 1x10^5 cells per well and incubated at 37°C for several days. LY-294002 (Sigma) was added where indicated at a concentration of 10 μM. After the indicated numbers of days supernatants were harvested for analysis of cytokines and cells were analyzed by flow cytometry.

CD4+ T cell proliferation analysis

Purified CD4+ T cells were labeled with the membrane dye carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular probes) for analysis of T cell proliferation. CD4+ T cells were harvested after three days of culture, counted and the reduction of signal of the CFSE dye per cell was analyzed by flow cytometry. Cell viability was assessed by resuspending cell pellets in Viaprobe dye (Pharmingen) followed by incubation at room temperature for 5 minutes before analysis. The total number of viable/non-viable cells after each round of division was calculated as follows. Cell populations that displayed similar dilution of the CFSE dye were gated, so that each gate represented cells that underwent an equal number of cell divisions. Within each gated population the percentage of Viaprobe negative and positive cells were distinguished. The absolute numbers of cells in the acquired gates were calculated by taking the respective percentage of the counted total T cell numbers. Cell debris and beads were excluded from analysis.

Flow cytometry

Cell suspensions were prepared, counted and stained with a panel of fluorochrome-conjugated Abs, all purchased at Pharmingen, except for αICOS Ab (C398.4A, eBioscience). The Ab stainings were performed in PBS/0.5% BSA and 0.1% azide supplemented with 2% goat serum. Dilutions of the staining Abs were added and cells were incubated at 4°C for 30 minutes. For intracellular Bcl-X_L staining,

cells were fixed in 96% ethanol and stained with α-Bcl-X_L Ab (Pharmingen) diluted 1:10 in 0.5% saponin (Sigma) solution. Stained cells were washed twice before analysis on a flow cytometer (Beckton Dickinson).

Multiplex Ligation-dependent Probe Amplification (MLPA)

Expression transcripts of apoptosis-related genes was analyzed by MLPA as described (19). Briefly, purified $CD4^+$ T cells were stimulated with αCD3, αCD3-αCD28 or αCD3-αICOS coated beads for 8 hours. mRNA was extracted and MLPA reactions were preformed. Results form all target genes were normalized to mRNA levels of the housekeeping gene β2-microglobulin.

mRNA analysis by quantitative PCR

Human primary T cells ($1x10^6$) were harvested and total mRNA was extracted using the RNAeasy mini kit (Qiagen) and was quantified by spectophotometry. Before first strand cDNA amplification, concentration was set at 200 ng/μl. Primers to detect 18S, 28S, Bcl-X_L, A1 and IL-2 were designed using Primer3 software (20). Real time PCR amplification and product detection was performed using the Light cycler (Roche) (18S, Bcl-X_L and A1) or the ABI Prism 7700 (Applied Biosystems) (28S, IL-2) as recommended by the manufacturer. A duplicate cDNA reaction was set up in which reverse transcriptase was left out and was used to detect the presence of genomic DNA. Results were normalized to 18S (for Bcl-X_L and A1 detection) or 28S RNA levels (for IL-2 detection) and relative expression was determined using the comparative cycle threshold method as recommended by the manufacturer. mRNA levels are expressed as *n*-fold difference relative to mRNA levels in unstimulated T cells.

Yeast-2-hybrid

cDNA from cytosolic tails of murine ICOS, CD28 and CTLA-4 were generated and cloned into $pPC97^{trp}$ vector, containing the GAL4 DNA binding domain. Oligo d(T) activated T cell cDNA library ($pPC86^{leu}$, Life Technologies) was transformed into yeast strain YGH1 (Clontech, Palo Alto, CA) and used to screen interacting proteins. Protein interactions were assessed by growth of colonies on leucine, tryptophan and histidine-depleted medium (-LTH), and β-galactosidase activity was assessed with a replica filter assay. cDNA sequences from interacting proteins were determined by blast analysis (NCBI).

Cell lysis, SDS-PAGE and Western blotting

Cells were lysed in 1% Nonidet P-40 lysis buffer. Lysates were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were probed with appropriate primary Abs and proteins were visualized with ECL^+ (Amersham, Piscataway, NJ). All primary Abs were obtained from (Cell Signaling Technology, Beverly, MA) and the anti-rabbit IgG-HRP secondary Abs was obtained from Calbiochem (San Diego, CA).

Production of high titer lentiviral vectors and transduction into $CD4^+$ T cells

Lentiviral vectors were produced after transfection of 293T human embryonic kidney cells cultured in RPMI 1640 (BioWhittaker, Rockville, MD), 10% FCS, 2 mM glutamine, and 100 IU/ml penicillin,

100 μg/ml streptomycin. Cells were seeded at 5 x 10^6 per T 150 tissue culture flask 24 h before transfection. All plasmid DNA was double-purified using a CsCl gradient. Cells were transfected with 7 μg of pMDG.1 (VSV-G envelope), 18 μg of pRSV.rev (HIV-1 Rev encoding plasmid), 18 μg of pMDLg/p.RRE (packaging plasmid), and 15 μg of pCLS transfer plasmid using Fugene 6 (Roche Molecular Biochemicals). Media was changed 6 h after transfection and the viral supernatant was harvested at 24 and 48 h post transfection (21). Viral particles were concentrated 10-fold by ultracentrifugation for 3 h at 28,000 rpm with a Beckman SW28 rotor (Beckman Coulter, Fullerton, CA). $CD4^+$ T cells were stimulated for 24 h with Ab-coated beads before infection. 300 to 500 μl (~5 x 10^7 - 5 x 10^8 IFU) of lentiviruses were incubated with the activated $CD4^+$ T cells on retronectin-coated 6-well plates at 37°C (22) and centrifuged at 1,200 x *g* for 2 h (23). The following day the medium was exchanged and the cells were expanded until they returned to near resting cell volume as measured by a Coulter Counter Multisizer II (Coulter, Hialeah, FL).

Results

ICOS induces cell viability in primary human T cells similar to CD28

CD28 is an important molecule for induction of primary T cell proliferation and T cell survival (1). Since ICOS is homologous to CD28, the role of ICOS in induction of cell survival of human $CD4^+$ T cells was determined. Therefore, purified CFSE-labeled human $CD4^+$ T cells were stimulated with Ab-coated beads. Expansion was determined by analyzing dilution of CFSE label by flow cytometry and counting absolute numbers of cells. Total numbers of viable cells that have gone through each round of division were distinguished simultaneously from non-viable cells by using Viaprobe as a cell death marker. This way cells that die after

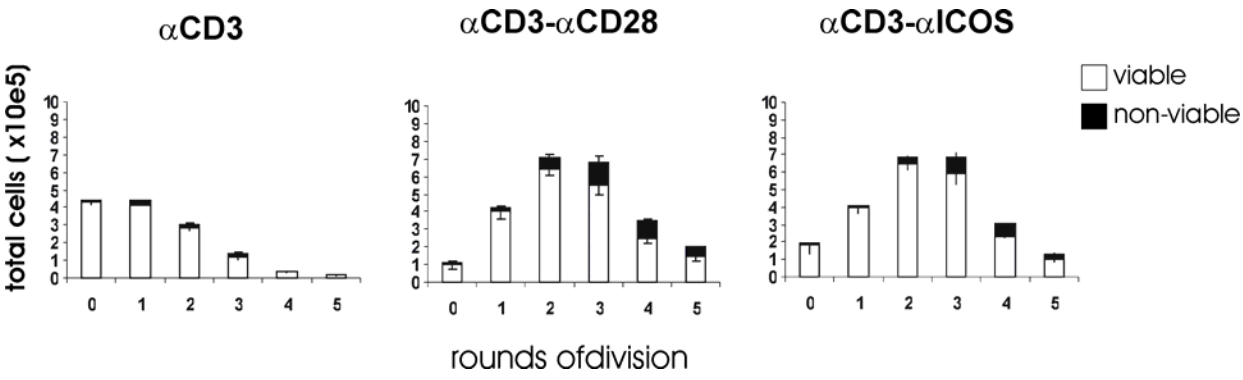


Figure 1. ICOS-mediated costimulation induces cell cycle progression and cell viability similar to CD28 in human $CD4^+$ T cells. $CD4^+$ T cells were isolated form peripheral blood and stimulated with Ab-coated beads as indicated. After 72 hrs cells were counted and CFSE profiles were determined by flow cytometry. The percentage of cells that underwent a number of divisions was determined and cell viability was simultaneously analyzed with Viaprobe as a marker for non-viable cells. Total viable (white bars) and non-viable (dark bars) T cell numbers which had undergone the number of divisions are indicated and were calculated using the percentage of cell with similar CFSE dilution profile of the total number of cells. Data are expressed as means ± SD.

going through one or more rounds of division can be distinguished from non-viable cells, which were not induced to proliferate. To minimize secondary effects of proliferation on cell death, both ICOS and CD28 signals were provided in addition to TCR signal (**Fig. 1**, αCD3) such that similar proliferation in T cells was induced (**Fig. 1**, compare αCD3-αCD28 and αCD3-αICOS). Under these conditions, ICOS-mediated costimulation results in equal viability of $CD4^+$ T cells that have gone through similar number of divisions compared to CD28-mediated costimulation (**Fig. 1A**, compare dark bars). Thus, under conditions at which ICOS and CD28 similarly propagate cell cycle progression, ICOS can maintain cell viability equally well as CD28.

ICOS-mediated costimulation enhances Bcl-X_L and A1 pro-survival genes

Next, it was investigated whether the induction of cell viability by ICOS- compared to CD28-mediated costimulation correlated with induction of pro- and anti-apoptotic genes. Therefore, RNA was isolated from stimulated $CD4^+$ T cells and expression of 45 transcripts of apoptosis-related genes was analyzed by *Multiplex Ligation-dependent Probe Amplification (MLPA)*. Of all genes tested, Noxa, Bcl-X_L and A1 mRNA were more than two fold enhanced after stimulation with αCD3 alone and further upregulated by αCD3-αICOS and αCD3-αCD28 after 8 hours (**Fig. 2A**). Noxa is a pro-apoptotic BH3-only protein that is induced in response to DNA damage (24). The relevance of upregulation of this apoptosis-inducing protein after TCR triggering remains to be elucidated. A1 (Bfl-1) is a pro-survival gene, which has previously been shown to be upregulated in T cells after αCD3-αCD28 stimulation (25). Subsequent mRNA analysis by quantitative PCR confirmed A1 mRNA induction by αCD3 and further upregulation by simultaneous stimulation with either αICOS or αCD28, which reached maximum expression after 2 hours after stimulation (**Fig. 2B** and data not shown). Furthermore, Bcl-X_L mRNA was detected as soon as 4 hrs in stimulated $CD4^+$ T cells and levels persisted after 24 hours (**Fig. 2B** and data not shown). Although upregulated by stimulation with αCD3 signal alone, Bcl-X_L mRNA levels were much more enhanced by additional ICOS and CD28-mediated costimulation. Moreover, the increase in mRNA levels resulted in increased Bcl-X_L protein levels, as determined by intracellular staining and flow cytometry (**Fig. 2C**). Interestingly, Bcl-X_L protein levels were equal under experimental conditions were ICOS and CD28-mediated costimulation induced similar proliferation of T cells. However, when CD28 was provided at higher concentration, Bcl-X_L levels were enhanced compared to ICOS-induced Bcl-X_L (data not shown). The Bcl-X_L upregulation showed to be independent of cell cycle progression since expression was enhanced in cells that had not divided and remained similar after proliferation (**Fig. 2D**, *left panels*). In conclusion, ICOS can induce anti-apoptosis genes A1 and Bcl-X_L in human $CD4^+$ T cells. In addition, Bcl-X_L upregulation occurs independently from ICOS and CD28-induced proliferation.

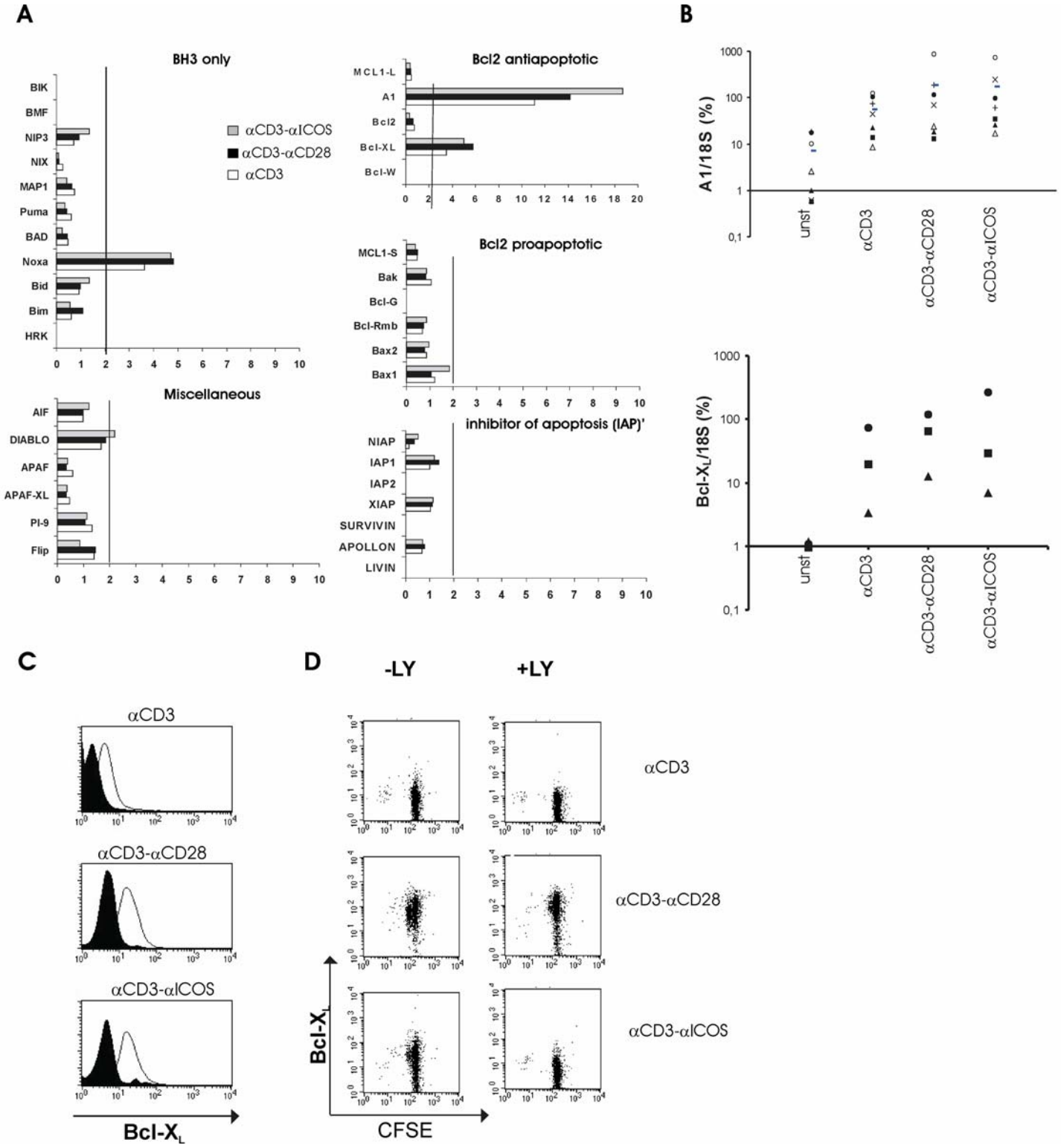


Figure 2. ICOS-mediated costimulation induces expression of A1 and Bcl-X_L. Purified $CD4^+$ T cells were stimulated with αCD3, αCD3-αCD28 and αCD3-αICOS coated beads, RNA was extracted and expression of apoptosis-related genes was analyzed with **(A)** MLPA analysis and **(B)** Quantitative PCR analysis. A1 and Bcl-X_L mRNA expression was determined after 2 hours and 24 hours respectively. Each symbol represents mRNA levels isolated from stimulated $CD4^+$ T cells derived from one donor. All RNA transcript levels are corrected for β2M (MLPA) or18S mRNA (quantitative PCR) and are relative to mRNA levels in unstimulated T cells. **(B)** Bcl-X_L protein expression. $CD4^+$ T cells were stimulated as in **(A)** and Bcl-X_L levels were determined after 48 hours by intracellular staining and flow cytometry. Results are representative of three independent experiments. In **(c)** cells were CFSE labeled prior to stimulation with αCD3, αCD3-αCD28^{hi} and αCD3-αICOS coated beads and Bcl-X_L expression/CFSE dilution was determined after 48 hours. LY-294002 was added to the cultures at a concentration of 10 μM. Results are representative of two independent experiments.

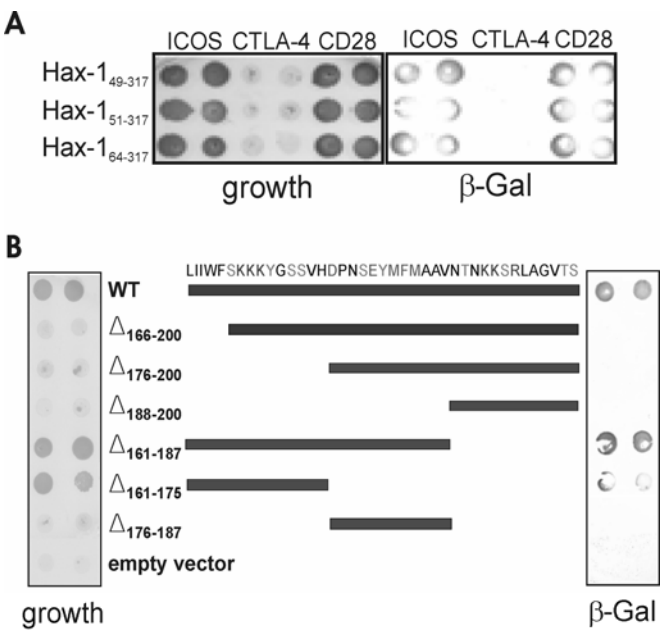
ICOS-mediated Bcl-X_L induction is inhibited by PI3K inhibition

The signal transduction mediator PI3K has been implicated in CD28 induced Bcl-X_L upregulation. Previous reports have shown that ICOS associates with and activates PI3K even to a greater extend than PI3K activation induced by CD28 (17).

We investigated the involvement of PI3K in ICOS-mediated Bcl-X_L upregulation in $CD4^+$ T cells by inhibition of PI3K activation with LY294002. Both ICOS-mediated proliferation and Bcl-X_L upregulation were reduced after addition of LY294002, while CD28-induced Bcl-X_L upregulation was only partially inhibited **(Fig. 2C**, *right panels*). Thus, PI3K is an important mediator of ICOS induced Bcl-X_L upregulation. Moreover, the partial inhibition observed for CD28 induced Bcl-X_L upregulation might implicate additional molecules are involved in this process.

Both ICOS and CD28 interact with Hax-1 in yeast

We next questioned whether other molecules, next to the p85 subunit of PI3K, could be recruited to ICOS and CD28 intracellular tail. Therefore, a yeast-2-hybrid screen was performed with the intracellular part of CD28 and ICOS. Three independent Hax-1 clones were found to associate with both the CD28 and ICOS intracellular tail in yeast, resulting in yeast growth and β-gal activity (**Fig. 3A**). Hax-1 is a 35 kDa cytoplasmatic protein, which was originally cloned as an HS-1-associating protein (26). While Hax-1 is ubiquitously expressed,



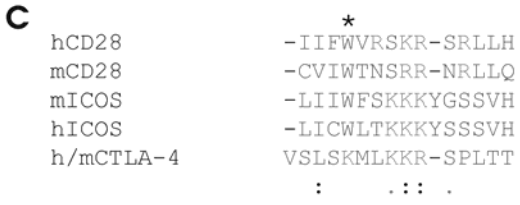


Figure 3. Hax-1 interacts with ICOS and CD28 in yeast. **(A)** Protein interactions of three independently picked up clones coding for the C-terminus of Hax-1 protein with the intracellular part of ICOS, CTLA-4 or CD28. Depicted are the size of the HAX-1 fragments (coding amino acids, *left panel*), growth on selective -LTH plates (*middle panels*), and β-galactosidase activity (*right panel*). Experiments were preformed three times with similar results. **(B)** Interaction of Hax-1 with ICOS tail mutants. Shown are growth on selective -LTH media and β-galactosidase activity of yeast cells containing Hax-1_{49-317} and the indicated ICOS tail mutants. Experiments using Hax-1_{51-317} or Hax-1_{64-317} yielded similar results. Depicted is representative of two independent experiments. **(C)** Comparison between AA sequence of proximal part of human (h) and murine (m) ICOS, CD28 and CTLA-4 intracellular tail. * = similar, **:** = homologous.

HS-1 expression is restricted to cells of hematopoetic origin and is involved in TCR and BCR signaling (27). The role of Hax-1 in T cells is unknown. Interestingly, Hax-1 did not associate with CTLA-4 (**Fig. 3A**). Deletion mutants of the ICOS intracellular tail showed that the binding domain of Hax-1 was within the proximal twelve AA and that the first five AA are crucial for association in yeast (**Fig. 3B**). Alignment of the first twelve AA of CD28 and ICOS intracellular tails revealed common tryptophan residue within the proximal five AA (**Fig. 3C**). Taken together, Hax-1 associates with both cytoplasmic ICOS and CD28 and the proximal five AA within the ICOS intracellular tail are indispensable for this association.

Hax-1 is involved in CD28 induced IL-2 production

We next determined whether ICOS or CD28 and Hax-1 could interact in mammalian cells. Therefore, Hax-1 was co-expressed with either ICOS or CD28 in 293T cells and co-localisation was investigated by confocal microscopy. Hax-1 was found to be expressed in the cytoplasm and no clear co-localisation with either ICOS or CD28 could be demonstrated in unstimulated cells. However, upon crosslinking of CD28, we found that Hax-1 was relocalized to the membrane (data not shown). This indicated that Hax-1 might play a role in the signaling pathway of CD28. Therefore, the functional consequence of Hax-1 association with CD28 was investigated by stimulating primary T cells in which Hax-1 protein levels were downregulated. To this end, primary human T cells were isolated and transfected with a vector containing Hax-1 RNAi oligo (siHax-1) linked to GFP reported gene by lentiviral transfection. Primary T cells were efficiently transduced, yielding reproducibly high levels of GFP (data not shown). Transduction of siHax-1 caused a significant downregulation of

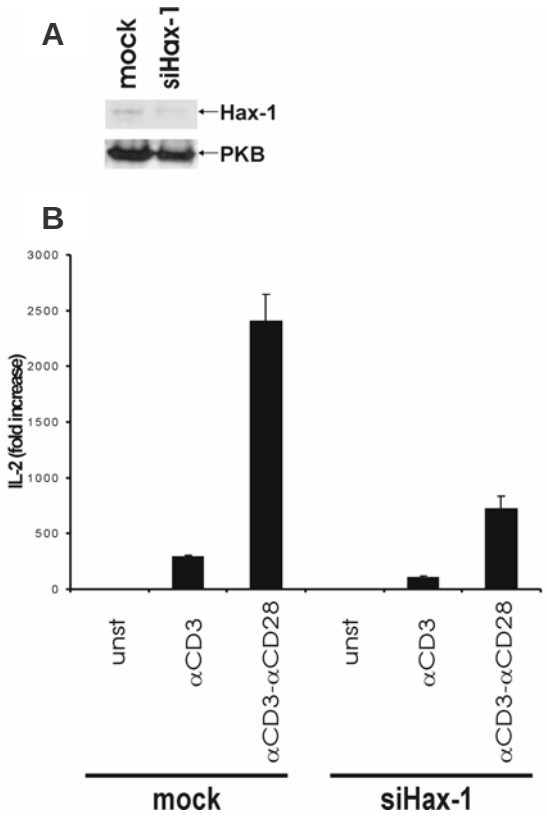


Figure 4. Hax-1 is involved in CD28-mediated IL-2 production. **(A)** Endogenous Hax-1 protein is downregulated in human CD4+ T cells. Primary CD4+ T cells were isolated and lentivirally transduced with vector encoding Hax-1 RNA-i oligo (siHax-1 or empty vector (mock). Cells were rested and restimulated with either αCD3 or αCD3-αCD28 coated beads. Total lysate was collected prior to restimulation. Endogenous levels of PKB were used as a loading control. **(B)** IL-2 production is reduced after αCD3-αCD28 stimulation in siHax-1 transfected cells. After 24 hours of stimulation mRNA was extracted and IL-2 levels were determined by quantitative PCR. Indicated is the relative increase of IL-2 mRNA as compared to levels in unstimulated CD4+ T cells. These experiments were performed twice with similar results.

endogenous Hax-1, although some residual Hax-1 was still expressed in the cells (**Fig. 4A**). Despite only partial downregulation of endogenous Hax-1, CD28-induced IL-2 production was clearly diminished after transfection of siHax-1 compared to mock transfected cells (**Fig. 4B**). Thus Hax-1 appears to be involved in CD28-mediated IL-2 production.

Discussion

CD28 is an important costimulatory molecule of T cells and in conjunction to TCR engagement CD28 ligation results in induction of proliferation, IL-2 production and cell survival. Since ICOS and CD28 are homologous, we questioned whether induction of survival is a common function between ICOS and CD28. It is demonstrated that ICOS propagates cell cycle progression while maintaining cell viability. Survival of T cells is regulated by the balance between pro- and anti-apoptotic proteins expressed in the cell (28). Members of the Bcl-2 family are expressed in naïve T cells, such as pro-apototic proteins Bax, Bak and Bim that contribute to induction of cell death in unstimulated cells (29, 30). Simultaneous expression of anti-apoptotic members, such as Bcl-2 (constitutively expressed in naïve T cells) and increased expression of Bcl-X_L by CD28 may counteract this (2, 31). We observed enhanced expression by both CD28 and ICOS of pro-survival member A1 as early as 2 hours after activation and Bcl-X_L upregulation in undivided cells. A1 may act in concert with Bcl-X_L prior to cell division to counterbalance the pro-apoptotic signals in resting cells. Similarly, simultaneous upregulation of A1 and Bcl-X_L might be a pre-requisite for ICOS-mediated induced cell cycle progression.

In the search for proteins contributing to CD28 and ICOS downstream signaling pathways we identified Hax-1 as a CD28- and ICOS-associating protein. Moreover, we demonstrate that Hax-1 is functionally involved in CD28-induced IL-2 production. Hax-1 is a cytoplasmatic protein, which has been found to associate with HS-1 (heamatopoeitic-specific molecule) (26). Several studies support an important role for HS1 in TCR signaling: not only is HS1 phosphorylated upon TCR triggering, it also has been found to associate with Lck, a Src family kinase critical for TCR signaling (27). In addition, HS1 defective T cells show impaired proliferation to various stimuli. Moreover, HS-1 was found to associate with Grb-2, a molecule that is recruited to the TCR upon ligation and, more importantly, a CD28 associating protein crucial for IL-2 production (32). Co-ligation of TCR and CD28 could therefore recruit Grb-2-HS1-Hax-1 in one complex necessary for optimal IL-2 production. Alternative functions described for Hax-1 are anti-apoptotic related, by virtue of its similarities with BH3 like proteins such as Nip3. Mice deficient for Hax-1, recently generated by J. Ihle, display T and B cell depletion 2-3 months after birth (personal communi-cation). However, we did not find increased cell death after RNAi in primary cells nor in jurkat cells, so a putative role for Hax-1 in T cell survival was not found under conditions where Hax-1 was partially downregulated (data not shown). Alternatively, Hax-1 functions downstream of CD28 through a yet unknown mechanism.

Preliminary data also suggest that Hax-1 associates with ICOS. In yeast, the binding domain pinpoints to the fist 10 AA

proximal in the ICOS tail. Sequence analysis revealed a common tryptophan residue in CD28 as well as ICOS intracellular tail. Alignment of other interacting proteins to Hax-1 did not reveal this common tryptophan, or any other consensus binding sequence. Co-localisation studies showed redistribution of Hax-1 after ICOS cross-linking, similar to CD28 crosslinking (data not shown). The role of Hax-1 in ICOS-mediated costimulation awaits elucidation.

In summary, ICOS and CD28 show similarities and differences in signaling pathways, although both induce similar pro-apoptosis genes in human $CD4^+$ T cells. Hax-1 might mediate one or more shared functions between both. Investigating CD28 and ICOS-mediated functions in Hax-$1^{-/-}$ T cells will provide more answers to the role of Hax-1 in CD28 and ICOS-mediated costimulation.

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References

1. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261:609-612.
2. Boise, L. H., A. J. Minn, P. J. Noel, C. H. June, M. A. Accavitti, T. Lindsten, and C. B. Thompson. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-X_L. *Immunity* 3:87-98.
3. Dahl, A. M., C. Klein, P. G. Andres, C. A. London, M. P. Lodge, R. C. Mulligan, and A. K. Abbas. 2000. Expression of bcl-X_L restores cell survival, but not proliferation off effector differentiation, in CD28-deficient T lymphocytes. *J.Exp.Med.* 191:2031-2038.
4. Rudd, C. E. and H. Schneider. 2003. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signaling. *Nat.Rev.Immunol.* 3:544-556.
5. Pages, F., M. Ragueneau, R. Rottapel, A. Truneh, J. Nunes, J. Imbert, and D. Olive. 1994. Binding of phosphatidyl-inositol-3-OH kinase to CD28 is required for T-cell signaling. *Nature* 369:327-329.
6. Okkenhaug, K., L. Wu, K. M. Garza, J. La Rose, W. Khoo, B. Odermatt, T. W. Mak, P. S. Ohashi, and R. Rottapel. 2001. A point mutation in CD28 distinguishes proliferative signals from survival signals. *Nat.Immunol.* 2:325-332.
7. Burr, J. S., N. D. Savage, G. E. Messah, S. L. Kimzey, A. S. Shaw, R. H. Arch, and J. M. Green. 2001. Cutting edge: distinct motifs within CD28 regulate T cell proliferation and induction of Bcl-X_L. *J.Immunol.* 166:5331-5335.
8. Schneider, H., Y. C. Cai, K. V. Prasad, S. E. Shoelson, and C. E. Rudd. 1995. T cell antigen CD28 binds to the GRB-2/SOS complex, regulators of p21ras. *Eur.J.Immunol.* 25:1044-1050.
9. Kim, H. H., M. Tharayil, and C. E. Rudd. 1998. Growth factor receptor-bound protein 2 SH2/SH3 domain binding to CD28 and its role in co-signaling. *J.Biol.Chem.* 273:296-301.
10. Harada, Y., E. Tanabe, R. Watanabe, B. D. Weiss, A. Matsumoto, H. Ariga, O. Koiwai, Y. Fukui, M. Kubo, C. H. June, and R. Abe. 2001. Novel role of phosphatidylinositol 3-kinase in CD28-mediated costimulation. *J.Biol.Chem.* 276:9003-9008.
11. Andres, P. G., K. C. Howland, A. Nirula, L. P. Kane, L. Barron, D. Dresnek, A. Sadra, J. Imboden, A. Weiss, and A. K. Abbas. 2004. Distinct regions in the CD28 cytoplasmic domain are required for T helper type 2 differentiation. *Nat.Immunol.* 5:435-442.
12. Walunas, T. L., D. J. Lenschow, C. Y. Bakker,

P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1:405-413.

13. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541-547.
14. Hutloff, A., A. M. Dittrich, K. C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos, and R. A. Kroczek. 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 397:263-266.
15. Yoshinaga, S. K., J. S. Whoriskey, S. D. Khare, U. Sarmiento, J. Guo, T. Horan, G. Shih, M. Zhang, M. A. Coccia, T. Kohno, A. Tafuri-Bladt, D. Brankow, P. Campbell, D. Chang, L. Chiu, T. Dai, G. Duncan, G. S. Elliott, A. Hui, S. M. McCabe, S. Scully, A. Shahinian, C. L. Shaklee, G. Van, T. W. Mak, and G. Senaldi. 1999. T-cell co-stimulation through B7RP-1 and ICOS. *Nature* 402:827-832.
16. Coyle, A. J., S. Lehar, C. Lloyd, J. Tian, T. Delaney, S. Manning, T. Nguyen, T. Burwell, H. Schneider, J. A. Gonzalo, M. Gosselin, L. R. Owen, C. E. Rudd, and J. C. Gutierrez-Ramos. 2000. The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. *Immunity* 13:95-105.
17. Parry, R. V., C. A. Rumbley, L. H. Vandenberghe, C. H. June, and J. L. Riley. 2003. CD28 and inducible costimulatory protein Src homology 2 binding domains show distinct regulation of phosphatidylinositol 3-kinase, Bcl-X_L, and IL-2 expression in primary human CD4 T lymphocytes. *J.Immunol.* 171:166-174.
18. Harada, Y., D. Ohgai, R. Watanabe, K. Okano, O. Koiwai, K. Tanabe, H. Toma, A. Altman, and R. Abe. 2003. A single amino acid alteration in cytoplasmic domain determines IL-2 promoter activation by ligation of CD28 but not inducible costimulator (ICOS). *J.Exp.Med.* 197:257-262.
19. Eldering, E., C. A. Spek, H. L. Aberson, A. Grummels, I. A. Derks, A. F. de Vos, C. J. McElgunn, and J. P. Schouten. 2003. Expression profiling via novel multiplex assay allows rapid assessment of gene regulation in defined signaling pathways. *Nucleic Acids Res.* 31:e153.
20. Rozen, S. and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. *Bioinformatics Methods and Protocols: Methods in Molecular Biology* 365-386.
21. Reiser, J. 2000. Production and concentration of pseudotyped HIV-1-based gene transfer vectors. *Gene Ther.* 7:910-913.
22. Pollok, K. E., H. Hanenberg, T. W. Noblitt, W. L. Schroeder, I. Kato, D. Emanuel, and D. A. Williams. 1998. High-efficiency gene transfer into normal and adenosine deaminase-deficient T lymphocytes is mediated by transduction on recombinant fibronectin fragments. *J.Virol.* 72:4882-4892.
23. O'Doherty, U., W. J. Swiggard, and M. H. Malim. 2000. Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding. *J.Virol.* 74:10074-10080.
24. Oda, E., R. Ohki, H. Murasawa, J. Nemoto, T. Shibue, T. Yamashita, T. Tokino, T. Taniguchi, and N. Tanaka. 2000. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 288:1053-1058.
25. Gonzalez, J., A. Orlofsky, and M. B. Prystowsky. 2003. A1 is a growth-permissive antiapoptotic factor mediating postactivation survival in T cells. *Blood* 101:2679-2685.
26. Suzuki, Y., C. Demoliere, D. Kitamura, H. Takeshita, U. Deuschle, and T. Watanabe. 1997. HAX-1, a novel intracellular protein, localized on mitochondria, directly

associates with HS1, a substrate of Src family tyrosine kinases. *J.Immunol.* 158:2736-2744.

27. Taniuchi, I., D. Kitamura, Y. Maekawa, T. Fukuda, H. Kishi, and T. Watanabe. 1995. Antigen-receptor induced clonal expansion and deletion of lymphocytes are impaired in mice lacking HS1 protein, a substrate of the antigen-receptor-coupled tyrosine kinases. *EMBO J.* 14:3664-3678.
28. Opferman, J. T. and S. J. Korsmeyer. 2003. Apoptosis in the development and maintenance of the immune system. *Nat.Immunol.* 4:410-415.
29. Lindsten, T., A. J. Ross, A. King, W. X. Zong, J. C. Rathmell, H. A. Shiels, E. Ulrich, K. G. Waymire, P. Mahar, K. Frauwirth, Y. Chen, M. Wei, V. M. Eng, D. M. Adelman, M. C. Simon, A. Ma, J. A. Golden, G. Evan, S. J. Korsmeyer, G. R. MacGregor, and C. B. Thompson. 2000. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol.Cell.* 6:1389-1399.
30. Bouillet, P., D. Metcalf, D. C. Huang, D. M. Tarlinton, T. W. Kay, F. Kontgen, J. M. Adams, and A. Strasser. 1999. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 286:1735-1738.
31. Noel, P. J., L. H. Boise, J. M. Green, and C. B. Thompson. 1996. CD28 costimulation prevents cell death during primary T cell activation. *J.Immunol.* 157:636-642.
32. Takemoto, Y., M. Furuta, M. Sato, P. R. Findell, W. Ramble, and Y. Hashimoto. 1998. Growth factor receptor-bound protein 2 (Grb2) association with hemopoietic specific protein 1: linkage between Lck and Grb2. *J.Immunol.* 161:625-630.

Chapter 5

A critical but differential contribution of CD28 and ICOS in the adjuvant activity of *Neisseria meningitidis* H44/76 LPS and *lpxL1* LPS

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Summary

The development of novel vaccines against *Neisseria meningitidis* recently gained momentum by the generation of a mutant form of lipopolysaccharide (LPS), *lpxL1* LPS, which has comparable adjuvant activity, but reduced endotoxic activity compared to wild type (H44/76) LPS. The costimulation requirements for optimal IgG production evoked by H44/76 LPS or *lpxL1* LPS were investigated *in vivo* by immunizing mice lacking the B7.1/2/CD28 or B7RP-1/ICOS pathway or lacking both pathways with outer membrane complexes of LPS-deficient bacteria supplemented with either H44/76 LPS or *lpxL1* LPS. Our experiments demonstrate that B7RP-1/ICOS costimulation predominates over B7.1/2/CD28 costimulation in the adjuvant function of *lpxL1* LPS, while ICOS and CD28 are equally important for the adjuvant activity of H44/76 LPS. The IgG response against *N. meningitidis* is solely dependent on CD28 and ICOS, since no IgG is detected in mice that are deficient for both B7.1/2 and ICOS. Thus we report differential costimulation requirements between H44/76 LPS and *lpxL1* LPS, while both forms of LPS evoke similar magnitudes of IgG response. This implicates that ICOS-mediated costimulation predominates in the adjuvant function of *lpxL1* LPS and that exploiting the B7RP-1/ICOS pathway could be beneficial to optimize vaccines against *N. meningitidis.*

Submitted

Introduction

Neisseria meningitidis group B is a human pathogen, which can cause severe diseases as meningitis and septic shock. Vaccines under development against *N. meningitidis* are based on outer membrane vesicles (OMV), which primarily consist of outer membrane proteins (1-3). Immunization studies with these vaccines have revealed that their immunogenicity is low. Improvement of the vaccines is thus required and could potentially be achieved by inclusion of *N. meningitidis* lipopolysaccharide (LPS), which has strong adjuvant activity (4). The endotoxic effects of LPS however are a major drawback and have limited the use of LPS in human vaccines so far.

The portion of LPS responsible for its biological activities has been established to reside in its lipid A part (**Fig. 1**) (5). We recently demonstrated that alteration of the fatty acyl composition of *N. meningitidis* lipid A by genetic modification of its lipid A biosynthesis pathway had profound effects on the biological activity of LPS (6). Insertional inactivation of the *lpxL1* gene, encoding one of the acyloxyacyl transferases, resulted in a penta-acylated LPS that displayed 100-fold reduction in endotoxin activity as compared to wild type H44/76 LPS. Interestingly, the adjuvant activity of *lpxL1* LPS was unaffected as demonstrated by its ability to restore the immunogenicity of outer membrane complexes (OMC) of a completely LPS-deficient *N. meningitidis* mutant (6, 7). These improved biological properties make mutant *lpxL1* LPS an interesting adjuvant for inclusion in vaccines.

The pathways that determine the adjuvant properties of *N. meningitidis* LPS are not well defined. The adjuvant activity of bacterial stimuli is believed to result from their capability to target toll like receptors (TLR) on dendritic cells (DC), which leads to maturation of the DC (8). During this process, expression of MHCII and costimulatory molecules such as the TNF family member CD40 and members of the

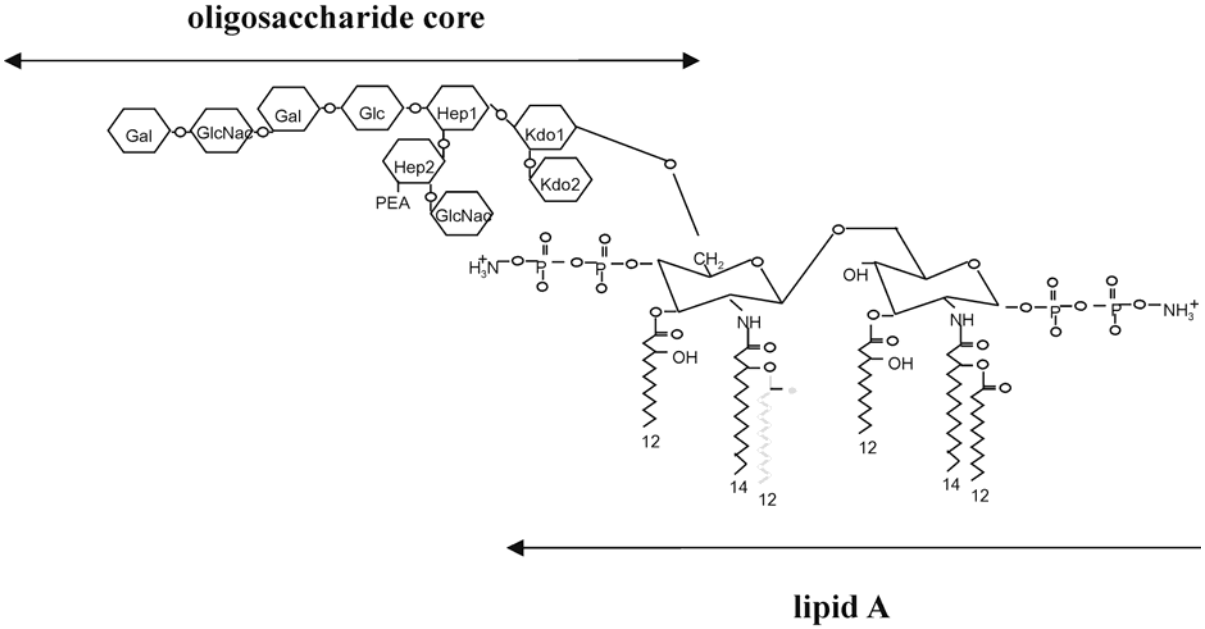

Figure 1. Schematic representation of wild type *N. meningitidis* LPS. Indicated in grey is the side chain, which is deleted in *lpxL1* LPS.

B7 costimulatory family is enhanced. The best studied members of the B7 family are B7.1 (CD80), B7.2 (CD86) and B7RP-1, which induce T cell activation and Th1/Th2 differentiation upon ligation with their counter receptors on T cells, CD28 and ICOS (for Inducible COStimulator) respectively (reviewed in ref. 9). Primed T cells in turn can provide the necessary help for B cells in antibody (Ab) production and Ab class switching. Studies in mice deficient for CD28 or ICOS have revealed that both molecules are necessary for profound humoral responses. More specifically, immune responses in the absence of ICOS or CD28 resulted in formation of smaller germinal centers and defective Ab class switching mainly towards IgG2a and IgG1 (10-14). It is crucial that the expression of costimulatory ligands and their receptors is tightly spatially and temporally tightly controlled, since aberrant costimulation has severe consequences for the outcome of a specific immune response.

As costimulatory molecules comprise an important link between the innate and adaptive immune systems, we addressed the regulation of expression of costimulatory molecules by different adjuvants. Several studies have shown that LPS-TLR4 interactions induce upregulation of B7.2 expression on DC (15), however the effect of LPS on B7RP-1 expression has not been well defined. Likewise, while B7.1 and B7.2 are upregulated by *lpxL1* LPS, the effect on B7RP-1 expression has not been described (16). More importantly, the functional consequences of the regulation of the B7.1/2 ligands on DC on the IgG production *in vivo* has not been investigated. This, together with the importance of B7 costimulation in the generation of humoral immune responses, prompted us to investigate whether B7.1-B7.2/CD28 and B7RP-1/ICOS interactions play a role in the adjuvant function of *N. meningitidis* H44/76 LPS and mutant *lpxL1* LPS. Our results demonstrate that ICOS and CD28 together are responsible for the IgG response against *N. meningitidis*, since no IgG could be detected in sera from B7.1/2$^{-/-}$ ICOS$^{-/-}$ mice. More importantly, it is shown that the contribution of ICOS to the IgG response evoked by *lpxL1* LPS predominates over the contribution of CD28.

Materials & Methods

Bacterial strains and growth conditions

The structures of the lipid A of *N. meningitidis* strain H44/76 and its *lpxL1* derivative used in this study are shown in **fig. 1**. All strains were grown overnight at 37°C on GC medium base (Difco Laboratories) supplemented with IsoVitaleX (Becton Dickinson) in a humid atmosphere containing 5% CO_2 or in liquid Meningococcal Medium as described (17). Bacterial suspensions were heat-inactivated for 30 min at 56°C.

Isolation of outer membrane complexes (OMCs)

Meningococci were grown in Meningococcal Medium, and OMCs were isolated by sarkosyl extraction (18). The protein content was determined by using the bicinchoninic acid protein assay reagent (Pierce Chemical Co.) with BSA as a standard. The protein composition of H44/76 LPS- and *lpxL1* LPS-containing OMCs was similar as analyzed by sodium dodecyl sulfate-polyacrylamide gel-

electrophoresis (SDS-PAGE) and staining with Coomassie Brilliant Blue.

Isolation of LPS

Meningococcal H44/76 wild type and *lpxL1* LPS mutant LPS were isolated by the hot phenol extraction method described by Westphal and Jann (19).

Animal strains

C57BL/6 CD28-deficient mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA) and C57BL/6 ICOS-deficient mice were kindly provided by R. Flavell (Yale University School of Medicine, New Haven) (20). C57BL/6 $ICOS^{-/-}$ mice were intercrossed with 129/SvS4Jae $B7.1/2^{-/-}$ mice (kindly provided by A. Sharpe, Brigham and Women's Hospital, Boston, MA) (21), generating $B7.1/2^{-/-}ICOS^{-/-}$ triple knockout mice and $B7.1/2^{+/-}ICOS^{-/-}$ littermates. All animal experiments were performed in compliance with institutional guidelines of AALAC and were approved by the institutional animal care and ethics committee.

Immunization of mice and serum analysis

Ten- to twelve-weeks old $CD28^{-/-}$, $ICOS^{-/-}$ or $B7.1/2^{-/-}ICOS^{-/-}$ mice and their wild type- and heterozygous littermates, were immunized subcutaneously on day 0, 14 and 28 with LPS-deficient OMCs (5 μg protein) supplemented or not with 1.25 μg purified H44/76 LPS or *lpxL1* LPS. Sera was collected at day 42 and antibody titers were determined for each individual serum against H44/76 whole cells in enzyme-linked immunosorbent assays (ELISAs) as described (22). Total IgG and IgG subclasses were determined using goat-anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3 and horseradisch peroxidase (HRP)-labeled conjugates (Southern Biotechnology Associates, Inc.).

Statistical analysis

A four-parameter curve fit was made for the optical density values obtained with serial dilutions of the sera, and the antibody titers were calculated as reciprocal dilutions that gave 50% of the maximum absorbance (= OD50). Before statistical analysis antibody titers were $\log_{10}$ converted, which normalized their distributions. Results were analyzed using a Student's *t* test (two-tailed). Differences between groups were considered statistically significant at the $p<0.05$ level.

Dendritic cell stimulation

Dendritic cells (DC) were cultured from bone marrow as described by Inaba *et al.* (23). At day 7 immature DCs were analyzed for cell surface expression of CD11c and CD11b by flow cytometry. Subsequently, DCs were stimulated with OMCs containing H44/76 LPS or *lpxL1* LPS (at an LPS concentration of 100 ng/ml) or LPS-deficient OMCs (at matching protein concentrations to H44/76 LPS or *lpxL1* LPS containing OMC). After 20 hours and 40 hours DCs were stained with a panel of fluorochrome-conjugated Abs as indicated, all purchased at PharMingen (PharMingen SanDiego, CA), except for B7RP-1-bio (eBiosciences). Non-specific FcR binding was prevented with FcR blocking Ab, clone 2.4G2 (Pharmingen). Fluorchrome labeled isotype controls were used as negative controls. Stained cells were analyzed by flow cytometry using a FACScalibur (BD Bioscience).

Results

B7.1/2 and B7RP-1 expression are differentially regulated in response to N. meningitidis *H44/76 LPS and* lpxL1 *LPS*

In search for new vaccines against *N. meningitidis* we have previously shown that the less toxic mutant *lpxL1* LPS maintained similar adjuvant capacity compared to its wild type counterpart H44/76 LPS and fully restores the immunogenicity of LPS-deficient OMC (7). To elucidate a putative difference in the contribution of B7 costimulatory pathways to the adjuvant activity of *N. meningitidis* H44/76 LPS versus *lpxL1* LPS, expression levels of B7.1, B7.2 and B7RP-1 on murine bone marrow derived DC were investigated (**Fig. 2**). Unstimulated immature DC expressed low levels of the CD28 ligand B7.1 and B7.2 and high levels of the ICOS ligand B7RP-1, which remained stable after culture in the absence of stimuli. Stimulation of DCs with H44/76 LPS OMC as well as with *lpxL1* LPS OMC induced similar upregulation of MHCII, a marker for DC maturation, while after stimulation with LPS-deficient OMC MHCII expression levels were unchanged compared to levels on unstimulated DCs

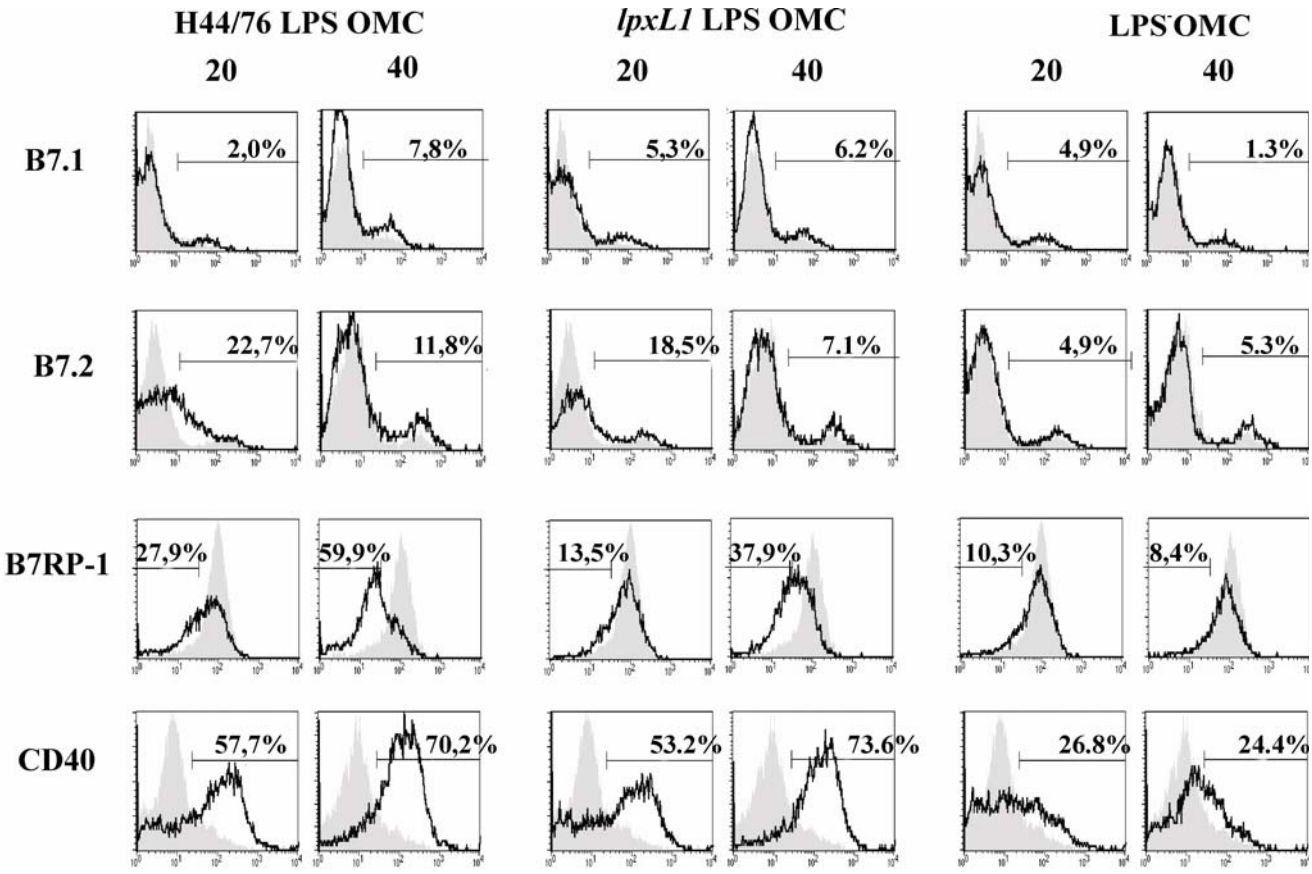

Figure 2. Differential regulation of B7 costimulatory molecules on DC by OMC *in vitro*. Bone marrow derived DC were either left unstimulated (shaded plot), or stimulated for 20 and 40 hours (black line) with OMC containing H44/76 LPS or *lpxL1* LPS or LPS-deficient OMC (LPS^-). Shown is the increase in percentages of positively stained cells for the indicated molecules compared to unstimulated cells. Results are a representative of two independent experiments.

(data not shown). As soon as 20 hrs after stimulation of DC with H44/76 LPS OMC expression of B7.2 was enhanced, while B7.1 was only slightly upregulated. In contrast, H44/76 LPS OMC induced downregulation of the ICOS ligand B7RP-1 on murine DC, which became most apparent after 40 hours of culture. More importantly, culturing DC in the presence of *lpxL1* LPS OMC also resulted in an enhanced B7.2 expression and a reduction of B7RP-1 expression, albeit to a lesser extend than in the presence of H44/76 LPS OMC. Expression of CD40, a costimulatory molecule of the TNF-related family, was similarly elevated on DC after stimulation in the presence H44/76 and *lpxL1* LPS OMC. H44/76 LPS and *lpxL1* LPS were the components responsible for the change in expression of B7.1/2 and B7RP-1 on DC, since the B7.1/2 expression levels on DC were unchanged after stimulation with the corresponding LPS-deficient OMC. Taken together, H44/76 LPS differentially regulated the expression of B7 costimulatory family members on DC; B7RP-1 was downregulated, while B7.1 and B7.2 were upregulated.

ICOS-mediated costimulation predominates in the adjuvant activity of lpxL1 *LPS*

Once we had observed that H44/76 LPS and *lpxL1* LPS induce differential expression of members of the B7 family members *in vitro*, we next investigated the functional requirement for B7.1/2/CD28 and B7RP-1/ICOS ligand-receptor pairs in the adjuvant activity of H44/76 LPS and *lpxL1* LPS *in vivo*. Therefore, wild type, CD28$^{-/-}$ and ICOS$^{-/-}$ mice were immunized with LPS-deficient OMC supplemented with either H44/76 LPS or *lpxL1* LPS, and serum IgG levels were determined by whole cell ELISA (**Fig. 3**). Mice were immunized with OMC containing 5 µg protein, since dose-response analysis revealed that serum IgG

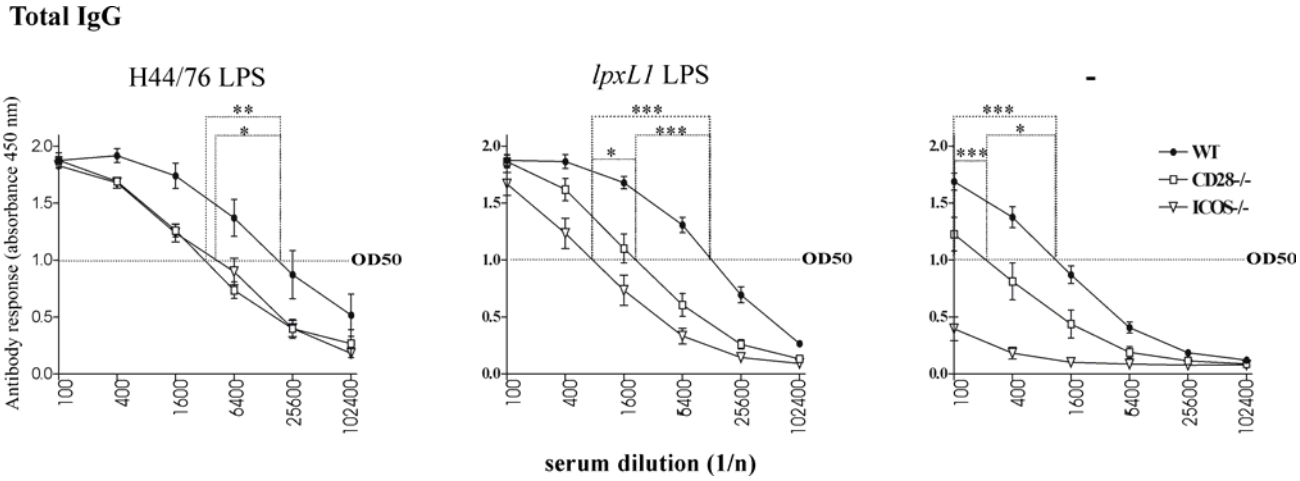

Figure 3. The IgG response induced by *lpxL1* LPS is more dependent on ICOS than on CD28. Wild type, CD28$^{-/-}$, or ICOS$^{-/-}$ mice were immunized with LPS-deficient OMC supplemented or not with H44/76 LPS, or *lpxL1* LPS. Sera were collected on day 42 and IgG titers were determined by whole-cell ELISA. Results are expressed as absorbance measured at 450 nm ± SEM. Indicated is OD50, which is the optical density at 50% of the maximal absorbance. Six to twelve mice were analyzed per group generated from at least two independent experiments; * $p<0.05$; ** $p<0.05$; *** $p<0.001$.

levels reached an optimum when mice were immunized with OMC containing 5 μg protein (unpublished observations). In wild type mice, immunization with LPS-deficient OMC alone evoked a minimal IgG response, which was diminished in CD28$^{-/-}$ mice and absent in ICOS$^{-/-}$ mice. In wild type mice, both H44/76 LPS and *lpxL1* LPS similarly increased the IgG levels induced by LPS-deficient OMC more than 20-fold, underscoring their comparable potent adjuvant activity as previously described (**Fig. 3**) (7). In CD28$^{-/-}$ mice, immunization in the presence of H44/76 LPS and *lpxL1* LPS induced a 4- to 5-fold lower IgG response as compared to wild type mice. Similarly, a 4- to 5-fold reduction in IgG serum levels was found when ICOS$^{-/-}$ mice were immunized in the presence of H44/76 LPS. In contrast, when these mice were immunized with LPS-deficient OMCs and *lpxL1* LPS a 17-fold reduction in the IgG response was found as compared to the response evoked in wild type mice. This indicates that the

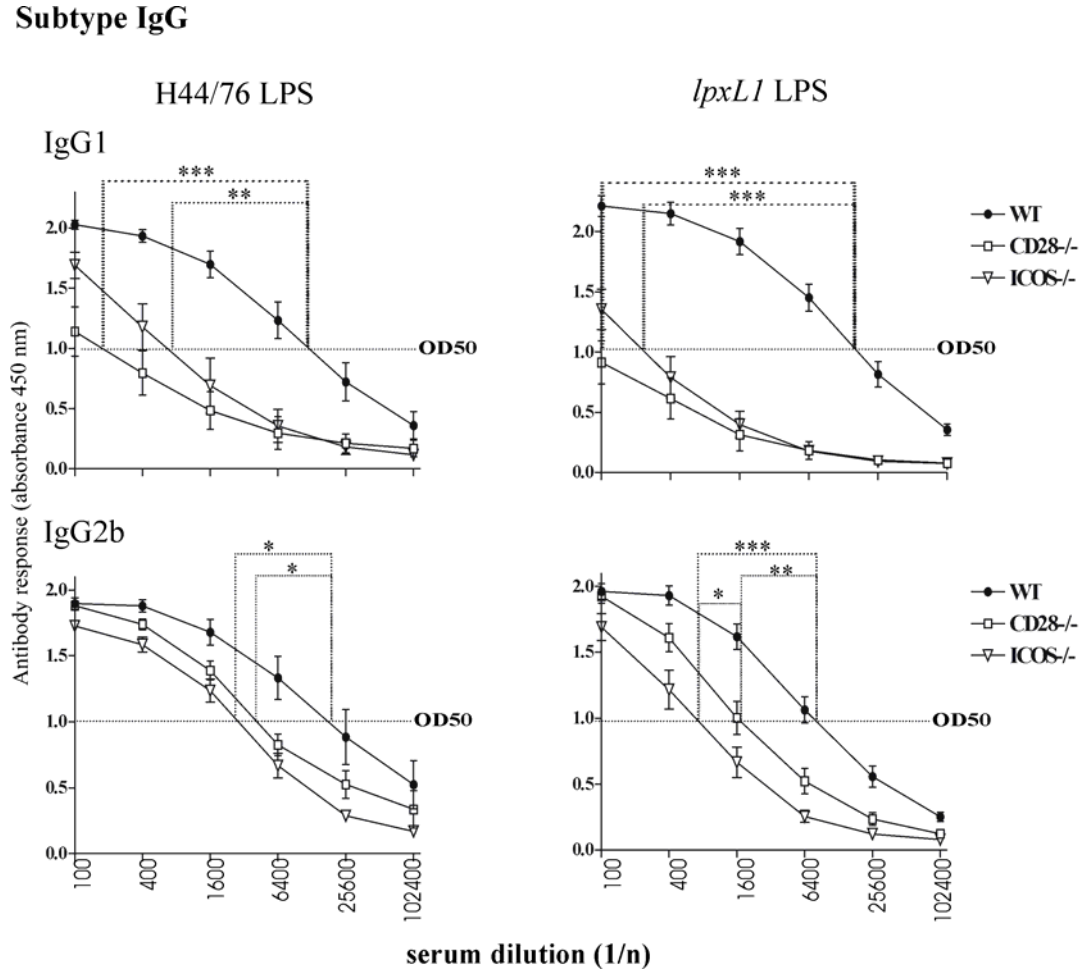

Figure 4. Contribution of CD28 and ICOS to OMC specific IgG1 and IgG2b subtype responses in the presence of H44/76 LPS or *lpxL1* LPS. Wild type, CD28$^{-/-}$ or ICOS$^{-/-}$ mice were immunized with LPS-deficient OMC supplemented with H44/76 LPS or *lpxL1* LPS. Sera were collected on day 42 and IgG1 and IgG2b titers were determined by whole cell ELISA. Results are expressed as absorbance measured at 450 nm ± SEM. Indicated is OD50, which is the optical density at 50% of the maximal absorbance. Six to twelve mice were analyzed per group generated from at least two independent experiments; * $p<0.05$; ** $p<0.05$; *** $p<0.001$.

adjuvant function of both H44/76 LPS and *lpxL1* LPS requires CD28-costimulation to the same extend, whereas the adjuvant activity of mutant *lpxL1* LPS is more dependent on ICOS than observed for H44/76 LPS.

LpxL1 *LPS adjuvant activity augments ICOS dependent IgG1 and IgG2b production*

For the development of effective vaccines against *N. meningitidis* it is important not only to elicit proper Ab titers, but also to enhance specific subclasses of Abs capable of inducing Ab-mediated effector functions necessary for immunity against this pathogen. Among different murine IgG subtypes, IgG2a and IgG2b are considered to be most important in the protection against *N. meningitidis* for their complement activating activity, while IgG1 production is less protective (24, 25). Since both ICOS and CD28 are reported to be crucial in isotype switching, we next determined the subclass distribution of the antibodies elicited in wild type, CD28$^{-/-}$ and ICOS$^{-/-}$ mice in response to LPS-deficient OMC supplemented with H44/76 LPS or *lpxL1* LPS (**Fig. 4**). Unfortunately, C57BL/6 mice are unable to produce high titers of IgG2a (26), which was therefore not included in further analysis. Furthermore, IgG3 and IgG2c levels were undetectable after immunization with both LPS-deficient OMC supplemented with H44/76 LPS or *lpxL1* LPS in all mouse strains tested (data not shown).

In wild type mice, the IgG1 and IgG2b Ab levels induced by immunization with *lpxL1* LPS and LPS-deficient OMC were not significantly different from IgG1 and IgG2b levels found in the presence of H44/76 LPS, demonstrating that these adjuvants elicited comparable subclass

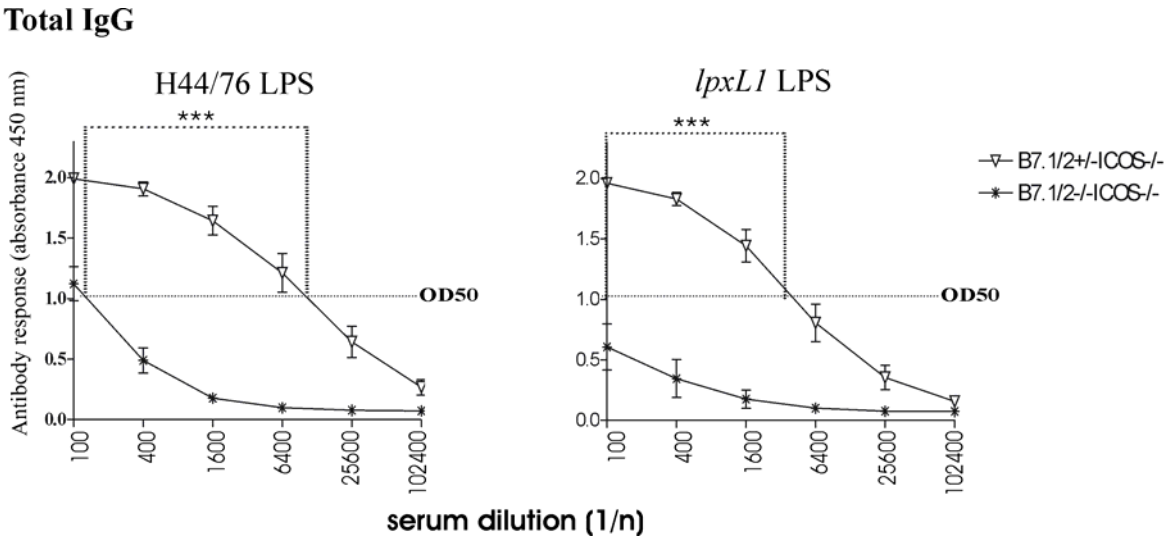

Figure 5. OMC specific IgG responses in the presence of H44/76 LPS or *lpxL1* LPS are completely dependent on CD28 and ICOS. B7.1/2$^{-/-}$ICOS$^{-/-}$ mice or B7.1/2$^{+/-}$ICOS$^{-/-}$ littermates were immunized with LPS-deficient OMC supplemented with H44/76 LPS or *lpxL1* LPS. Sera were collected on day 42 and total IgG titers were determined by whole-cell ELISA. Results are expressed as absorbance measured at 450 nm ± SEM. Indicated is OD50, which is the optical density at 50% of the maximal absorbance. Seven to eight mice were analyzed per group generated from at least two independent experiments; *** $p<0.001$.

distributions. Yet, the induction of class switching of *lpxL1* LPS and H44/76 LPS differed with respect to the costimulation requirements. ICOS and CD28 were indispensable for IgG1 class switching for both H44/76 LPS and *lpxL1* LPS. In contrast, IgG2b class switching was more dependent on ICOS in the case of *lpxL1* LPS, as concluded from a 14-fold reduction of serum IgG found after immunization of $ICOS^{-/-}$ mice compared to wild type mice, whereas in $CD28^{-/-}$ mice IgG2b levels were only 5-fold reduced. Taken together, while ICOS and CD28 show overlapping functions in class switching, ICOS is more important in class switching towards the protective subclass IgG2b after immunization in the presence of *lpxL1* LPS compared to CD28.

ICOS and CD28 are both required and sufficient for the adjuvant activity of lpxL1 *LPS*

To test whether residual IgG production in $ICOS^{-/-}$ mice after immunization in the presence of *lpxL1* LPS could be attributed to CD28, vaccinations using mice deficient for both the B7.1/2/CD28 and B7RP-1/ICOS and pathways were performed. Therefore, serum IgG levels were determined in $B7.1/2^{-/-}ICOS^{-/-}$ mice and $B7.1/2^{+/-}/ICOS^{-/-}$ littermates immunized with LPS-deficient OMC in the presence of either H44/76 LPS or *lpxL1* LPS. Interestingly, no serum IgG could be detected in $B7.1/2^{-/-}ICOS^{-/-}$ deficient mice after immunization in the presence of H44/76 LPS or *lpxL1* LPS (**Fig. 5**). Also, neither IgG1 nor IgG2b could be detected in $B7.1/2^{-/-}ICOS^{-/-}$ mice (data not shown). In $B7.1/2^{+/-}ICOS^{-/-}$ mice on mixed C57BL/6-129/SvS4Jae background total IgG levels after immunization were slightly higher (**Fig. 5**) as compared to levels in $ICOS^{-/-}$ mice on C57BL/6 background (**Fig. 3**), which reflected an overall higher IgG levels that could be evoked in 129/SvS4Jae wild type mice (data not shown). Apparently, B7.1/2/CD28 and B7RP-1/ICOS costimulatory pathways together are indispensable for the adjuvant activity of both H44/76 LPS and *lpxL1* LPS.

Discussion

The inclusion of LPS into OMV for vaccination against *Neisseria meningitidis* group B is desirable for it's high adjuvant activity, but restricted because of the toxic side effects of LPS. Fortunately, a less toxic variant of LPS was recently generated, *lpxL1* LPS, which has equally potent adjuvant activity compared to wild type H44/76 LPS in terms of IgG production (7). Despite similar adjuvant activity between H44/76 LPS and *lpxL1* LPS, we here report differences in their costimulation requirements. While H44/76 LPS and *lpxL1* LPS require CD28 to the same extend, ICOS is much more important in the adjuvant activity of *lpxL1* LPS compared to wild type LPS. This correlated with the availability of their respective ligand on DCs to a certain degree. Namely, B7RP-1 expression on DCs continued to be more pronounced after *lpxL1* LPS stimulation compared to H44/76 LPS stimulation. In contrast, the difference in B7.2 expression induced by H44/76 LPS compared to *lpxL1* LPS did not correspond with a more CD28 dependent response after immunization with the respective agents *in vivo*. Either minimal B7.2 expression on DC is sufficient to have maximum CD28 dependent costimulation or B7.1/2 expression on other APCs also contribute to the response after immunization *in vivo*.

The change in lipid A part as done in *lpxL1* LPS diminished the B7.1/2 upregulation and especially downregulation of B7RP-1 as was observed for H44/76 LPS, while CD40 expression was similar between the two forms. LPS exerts its effect on DC via binding to the TLR4/MD2/CD14 complex, which can signal via the Myd88-dependent or Myd88-independent TRIF/TRAM-dependent pathway (8). It has previously been demonstrated that LPS-mediated up regulation of B7.2 and CD40 on DC occurs via a MyD88-independent TRIF-mediated pathway downstream of TLR4 (27), whereas up regulation of B7RP-1 on macrophages by LPS primarily depends on the MyD88-dependent pathway (28). Furthermore, it has been shown that signaling of *N. meningitidis* H44/76 LPS is mediated via the TLR4/MD2 pathway (29). Because the composition of the lipid A part of LPS determines the signaling pathway that is preferentially used (30), it is conceivable that the mutation in the lipid A region, as done in *lpxL1* LPS, alters the interaction with TLR4, affecting MyD88-dependent and -independent signaling and consequently leading to differential regulation of B7.1/2 and B7RP-1.

The restored immunogenicity of LPS-deficient OMC supplemented with H44/76 LPS or *lpxL1* LPS could be due to generation of Abs against LPS itself. However, we have previously shown that no LPS-specific Abs could be detected in the sera of mice immunized with LPS-deficient OMC supplemented with H44/76 LPS (7). Based on these results it is likely that both H44/76 LPS and *lpxL1* LPS have a major function in adjuvant activity rather than an immunogenic function.

When mice were immunized with LPS-deficient OMC alone a minimal IgG response was still apparent, which was dependent on the presence of CD28 and ICOS. We and others have previously shown that porins, such as PorA and PorB, are the major antigenic components in OMC, which in the case of LPS-deficient OMC accounts for the induced IgG response after immunization (7, 17, 31). A CD28-dependent IgG response against neisserial porins has previously been demonstrated by Wetzler and coworkers (32). Unlike the low levels of B7.1 and B7.2 resulting from stimulation with OMC in the absence of LPS, ICOS ligand B7RP-1 remained highly expressed on the surface of DC. This could explain that next to CD28, ICOS is indispensable for IgG production against OMC in the absence of LPS. Therefore, one could speculate that ICOS is important for the immune response against Gram-positive bacteria. Whether patients with an ICOS defect are more sensitive to infection with Gram-positive bacteria compared to Gram-negative bacteria is a point of interest and remains to be investigated.

Both CD28 and ICOS have been shown to be important molecules for germinal center formation and isotype switching. In many studies both CD28 and ICOS are reported to be indispensable for isotype switching towards IgG1, which is in accordance with the role of ICOS and CD28 in Th2 differentiation (10, 11, 13, 14, 21). Consistent with this, either CD28 or ICOS could account for most of the IgG1 response after vaccination with OMC in the presence of either H44/76 LPS or *lpxL1* LPS, showing overlapping function. In contrast, ICOS was more important than CD28 for IgG2b

production after immunization in the presence of *lpxL1* LPS, although OMC specific IgG2b response could still occur in the absence of either ICOS or CD28.

The LPS adjuvant activity as assayed by serum IgG production is demonstrated to be completely dependent on the presence of both B7.1/2/CD28 and B7RP-1/ICOS ligand/receptor pair *in vivo*. These data are in accordance with data reported by Suh *et al.*, which also show that double mutation of the CD28 and ICOS pathways leads to profound defects in the IgG response against T-dependent antigen (11). The relative contribution of each ligand-receptor pair to the IgG response in vivo can be directly compared in the study described here. Our observation that ICOS plays a more critical role than CD28 for the IgG response after vaccination in the presence of *lpxL1* LPS suggests that there is an ICOS-dependent IgG response, which is CD28-independent.

This study has revealed an important role for ICOS in vaccination protocols. We have demonstrated that ICOS-B7RP-1 interaction is crucial in the eliciting of an Ab response against *N. meningitidis* in addition to the well-characterized B7.1/2-CD28 interaction. This has broad implications for our understanding of the differential function of the various members of the B7 costimulatory family and suggests that ICOS could be more critical in enhancing B cell responses. Interestingly, previous studies showed that continuous stimulation of ICOS by it's ligand B7RP-1 caused increased serum IgG production, especially of the Th1 related IgG2a subtype, which was less effective when CD28 was stimulated *in vivo* (33, 34). Studies in which we use an agonistic anti-ICOS Ab *in vivo* to enhance immune responses against *N. meningitidis* are currently on their way in our laboratory. Future studies will be aimed at comparing vaccination responders and non-responders with regard to ICOS and B7RP-1 expression in order to investigate the function of the B7RP-1/ICOS pathway more specifically in healthy human donors. These investigations could provide us with new information for design of better vaccines.

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References

1. Sierra, G. V., H. C. Campa, N. M. Varcacel, I. L. Garcia, P. L. Izquierdo, P. F. Sotolongo, G. V. Casanueva, C. O. Rico, C. R. Rodriguez, and M. H. Terry. 1991. Vaccine against group B *Neisseria meningitidis*: protection trial and mass vaccination results in Cuba. *NIPH Ann.* 14:195-207.
2. Fredriksen, J. H., E. Rosenqvist, E. Wedege, K. Bryn, G. Bjune, L. O. Froholm, A. K. Lindbak, B. Mogster, E. Namork, and U. Rye. 1991. Production, characterization and control of MenB-vaccine "Folkehelsa": an outer membrane vesicle vaccine against group B meningococcal disease. *NIPH Ann.* 14:67-79.
3. van der Ley, P. and J. T. Poolman. 1992. Construction of a multivalent meningococcal vaccine strain based on the class 1 outer membrane protein. *Infect.Immun.* 60:3156-3161.

4. Johnson, A. G. 1994. Molecular adjuvants and immunomodulators: new approaches to immunization. *Clin.Microbiol.Rev.* 7:277-289.
5. Homma, J. Y., M. Matsuura, S. Kanegasaki, Y. Kawakubo, Y. Kojima, N. Shibukawa, Y. Kumazawa, A. Yamamoto, K. Tanamoto and T. Yasuda. 1985. Structural requirements of lipid A responsible for the functions: a study with chemically synthesized lipid A and its analogues. *J.Biochem.(Tokyo)* 98:395-406.
6. van der Ley, P., L. Steeghs, H. J. Hamstra, J. ten Hove, B. Zomer, and L. van Alphen. 2001. Modification of lipid A biosynthesis in *Neisseria meningitidis* lpxL mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity. *Infect.Immun.* 69:5981-5990.
7. Steeghs, L., B. Kuipers, H. J. Hamstra, G. Kersten, L. van Alphen, and P. van der Ley. 1999. Immunogenicity of outer membrane proteins in a lipopoly-saccharide-deficient mutant of *Neisseria meningitidis*: influence of adjuvants on the immune response. *Infect.Immun.* 67:4988-4993.
8. Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat.Immunol.* 2:675-680.
9. Greenwald, R. J., G. J. Freeman, and A. H. Sharpe. 2005. The B7 family revisited. *Annu.Rev.Immunol.* 23:515-548.
10. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261:609-612.
11. Suh, W. K., A. Tafuri, N. N. Berg-Brown, A. Shahinian, S. Plyte, G. S. Duncan, H. Okada, A. Wakeham, B. Odermatt, P. S. Ohashi, and T. W. Mak. 2004. The inducible costimulator plays the major costimulatory role in humoral immune responses in the absence of CD28. *J.Immunol.* 172:5917-5923.
12. Dong, C., U. A. Temann, and R. A. Flavell. 2001. Cutting edge: critical role of inducible costimulator in germinal center reactions. *J.Immunol.* 166:3659-3662.
13. Tafuri, A., A. Shahinian, F. Bladt, S. K. Yoshinaga, M. Jordana, A. Wakeham, L. M. Boucher, D. Bouchard, V. S. Chan, G. Duncan, B. Odermatt, A. Ho, A. Itie, T. Horan, J. S. Whoriskey, T. Pawson, J. M. Penninger, P. S. Ohashi, and T. W. Mak. 2001. ICOS is essential for effective T-helper-cell responses. *Nature* 409:105-109.
14. McAdam, A. J., R. J. Greenwald, M. A. Levin, T. Chernova, N. Malenkovich, V. Ling, G. J. Freeman, and A. H. Sharpe. 2001. ICOS is critical for CD40-mediated antibody class switching. *Nature* 409:102-105.
15. Kaisho, T., O. Takeuchi, T. Kawai, K. Hoshino, and S. Akira. 2001. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J.Immunol.* 166:5688-5694.
16. Steeghs, L., J. Tommassen, J. H. Leusen, J. G. van de Winkel, and P. van der Ley. 2004. Teasing apart structural determinants of 'toxicity' and 'adjuvanticity': implications for meningococcal vaccine development. *J.Endotoxin.Res.* 10:113-119.
17. Wetzler, L. M., Y. Ho, and H. Reiser. 1996. Neisserial porins induce B lymphocytes to express costimulatory

B7-2 molecules and to proliferate. *J.Exp.Med.* 183:1151-1159.

18. van der Ley, P., J. E. Heckels, M. Virji, P. Hoogerhout, and J. T. Poolman. 1991. Topology of outer membrane porins in pathogenic *Neisseria* spp. *Infect.Immun.* 59:2963-2971.
19. Westphal, O. and J. K. Jann. 1965. Bacterial lipopolysaccharide extraction with phenol-water and further application of the procedure. *Methods Carbohydr.Chem.* 5:83-91.
20. Dong, C., A. E. Juedes, U. A. Temann, S. Shresta, J. P. Allison, N. H. Ruddle, and R. A. Flavell. 2001. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 409:97-101.
21. Borriello, F., M. P. Sethna, S. D. Boyd, A. N. Schweitzer, E. A. Tivol, D. Jacoby, T. B. Strom, E. M. Simpson, G. J. Freeman, and A. H. Sharpe. 1997. B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. *Immunity* 6:303-313.
22. abdillahi, H. P. J. 1987. Whole cell elisa for typing *Neisseria meningitides* with monoclonal antibodies. *FEMS Microbiol. Lett.* 48 :367-371.
23. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/ macrophage colony-stimulating factor. *J.Exp.Med.* 176:1693-1702.
24. Holst, J., B. Feiring, J. E. Fuglesang, E. A. Hoiby, H. Nokleby, I. S. Aaberge, and E. Rosenqvist. 2003. Serum bactericidal activity correlates with the vaccine efficacy of outer membrane vesicle vaccines against *Neisseria meningitidis* serogroup B disease. *Vaccine* 21:734-737.
25. Ey, P. L., G. J. Russell-Jones, and C. R. Jenkin. 1980. Isotypes of mouse IgG--I. Evidence for 'non-complement-fixing' IgG1 antibodies and characterization of their capacity to interfere with IgG2 sensitization of target red blood cells for lysis by complement. *Mol.Immunol.* 17:699-710.
26. Jouvin-Marche, E., M. G. Morgado, C. Leguern, D. Voegtle, F. Bonhomme, and P. A. Cazenave. 1989. The mouse Igh-1a and Igh-1b H chain constant regions are derived from two distinct isotypic genes. *Immunogenetics* 29:92-97.
27. Hoebe, K., E. M. Janssen, S. O. Kim, L. Alexopoulou, R. A. Flavell, J. Han, and B. Beutler. 2003. Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways. *Nat.Immunol.* 4:1223-1229.
28. Zhou, Z., K. Hoebe, X. Du, Z. Jiang, L. Shamel, and B. Beutler. 2005. Antagonism between MyD88- and TRIF-dependent signals in B7RP-1 up-regulation. *Eur.J.Immunol.* 35:1918-1927.
29. Pridmore, A. C., D. H. Wyllie, F. Abdillahi, L. Steeghs, L. P. van der, S. K. Dower, and R. C. Read. 2001. A lipopolysaccharide-deficient mutant of *Neisseria meningitidis* elicits attenuated cytokine release by human macrophages and signals via toll-like receptor (TLR) 2 but not via TLR4/MD2. *J.Infect.Dis.* 183:89-96.
30. Zughaier, S. M., S. M. Zimmer, A. Datta, R. W. Carlson, and D. S. Stephens. 2005. Differential induction of the toll-like receptor 4-MyD88-dependent and -

independent signaling pathways by endotoxins. *Infect.Immun.* 73:2940-2950.

31. Al Bader, T., K. A. Jolley, H. E. Humphries, J. Holloway, J. E. Heckels, A. E. Semper, P. S. Friedmann, and M. Christodoulides. 2004. Activation of human dendritic cells by the PorA protein of *Neisseria meningitidis*. *Cell Microbiol.* 6:651-662.
32. Mackinnon, F. G., Y. Ho, M. S. Blake, F. Michon, A. Chandraker, M. H. Sayegh, and L. M. Wetzler. 1999. The role of B/T costimulatory signals in the immuno-potentiating activity of neisserial porin. *J.Infect.Dis.* 180:755-761
33. Yoshinaga, S. K., J. S. Whoriskey, S. D. Khare, U. Sarmiento, J. Guo, T. Horan, G. Shih, M. Zhang, M. A. Coccia, T. Kohno, A. Tafuri-Bladt, D. Brankow, P. Campbell, D. Chang, L. Chiu, T. Dai, G. Duncan, G. S. Elliott, A. Hui, S. M. McCabe, S. Scully, A. Shahinian, C. L. Shaklee, G. Van, T. W. Mak, and G. Senaldi. 1999. T-cell co-stimulation through B7RP-1 and ICOS. *Nature* 402:827-832.
34. Guo, J., M. Stolina, J. V. Bready, S. Yin, T. Horan, S. K. Yoshinaga, and G. Senaldi. 2001. Stimulatory effects of B7-related protein-1 on cellular and humoral immune responses in mice. *J.Immunol.* 166:5578-5584.

Chapter 6

CD28/CTLA4 double deficient mice demonstrate crucial role for B7 co-stimulation in the induction of lower airways disease

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Summary

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$^{-/-}$). In the present study, it was investigated if T-cell co-stimulation *via* the putative B7-1/B7-2 receptor plays a role in the induction of Th2-mediated asthma manifestations 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* and up-regulated levels of serum OVA-specific IgE compared with the situation shortly before OVA challenge. In addition, eosinophil numbers and IL-5 levels 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 manifestations in B7-1/B7-2$^{-/-}$ mice. Importantly, also CD28/CTLA4$^{-/-}$ mice showed no asthma manifestations upon OVA sensitization and 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 manifestations in this murine model and, conversely, that CD28 signaling is crucial.

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Introduction

Allergic asthma is a complex inflammatory disease of the airways characterized by reversible airflow obstruction, persistent airway hyperresponsiveness to broncho-spasmogenic stimuli such as methacholine, chronic eosinophilic airway inflammation, and airway remodeling (1). 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 (2). 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) (3-5). 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 (6). 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 features of allergic airway sensitization (7-12).

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 (13). 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 (14-15).

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 features of allergic airway sensitization can be induced in B7-1/B7-2$^{-/-}$ mice (16), nor in wild-type mice treated with mAbs against both B7-1 and B7-2 throughout the antigen sensitization and challenge period (17). 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) (17-18) and the priming of lymphocytes in the lungs, spleen and lymph nodes (17-19). These data on residual T-cell activation in CD28$^{-/-}$ mice but not in B7-1/B7-2$^{-/-}$ mice (16), 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 features of allergic airway sensitization. However, the failure to induce these symptoms in CD28$^{-/-}$ mice might also be due to negative signaling by CTLA4 (20-22) 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*. (16), we failed to induce any of these features of allergic airway sensitization 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 features of allergic airway sensitization 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 (17, 19, 23).

Materials & Methods

Animals

Specific pathogen-free wild-type BALB/c mice were obtained from Charles River (Maastricht, the Netherlands). BALB/c CD28/CTLA4$^{-/-}$ mice (13) 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 week-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 i.p. injections of 10 μg OVA adsorbed onto 2.25 mg aluminium hydroxide (alum; ImjectAlum, Pierce, Rockford, IL, USA) in 0.1 ml pyrogen-free saline on days 0 and 7. On days 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 days 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 (P_{enh}), 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 P_{enh} 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 (three days before challenge) approximately 0.25 ml blood was recovered from mice by an incision in the tail vein. After measurement of airway responsiveness *in vivo* at 24 h after the last OVA aerosol challenge on day 42, mice were killed 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 microtitre 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 PBS. The next day, the ELISA was performed at room temperature using ELISA buffer (PBS containing 0.5% 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 -described above 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. Colour 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 OD 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.

Analyzis of the BALF

BAL was obtained 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 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 Assistant 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 AG, Düdingen, Switzerland). Per cytospin, at least 200 cells were counted and differentiated into mononuclear cells (monocytes, macrophages and lympho-cytes), 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 described above 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 leucocytes. 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 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 days 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 millilitre BALF and of the 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 ± 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, homoscedastic). 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 days 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 **fig. 1**, all three OVA-sensitized BALB/c strains, demonstrated a dose-dependent increase in P_{enh}, 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. Upon OVA aerosol challenge, wild-type mice developed airway hyperresponsiveness because as 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 ($P < 0.05$, **Fig. 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 (**Fig. 2**). In contrast, as in naïve wild-type mice (data not shown), only

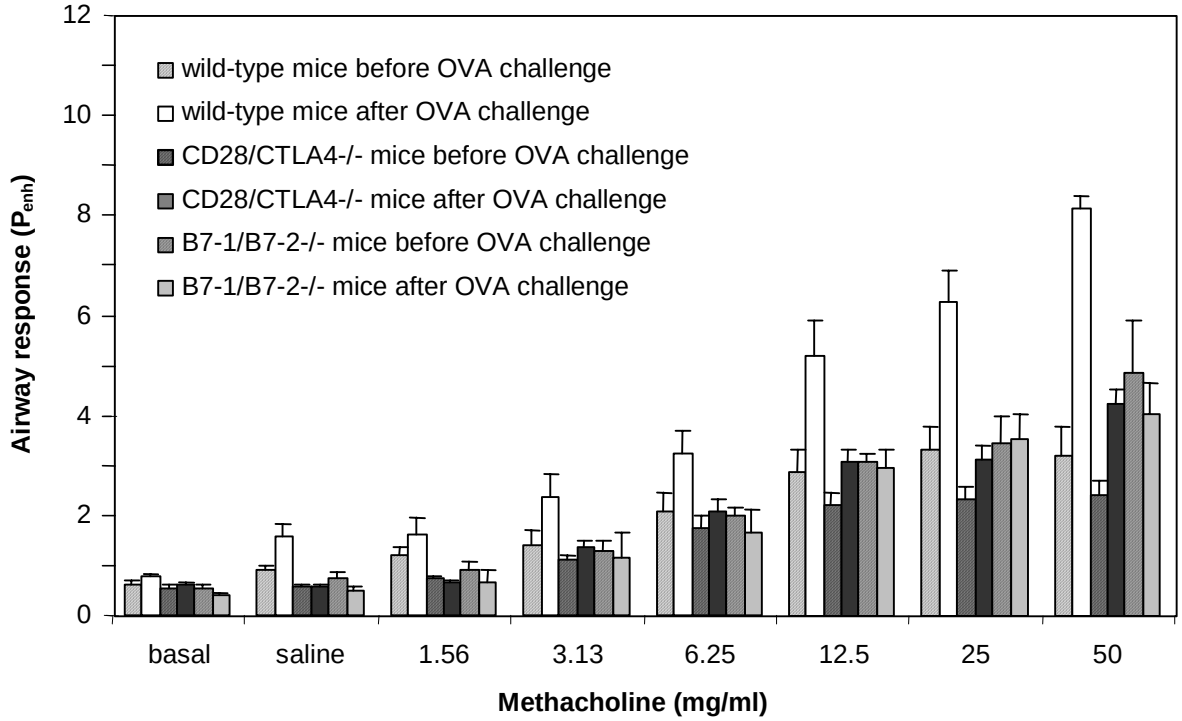

Figure 1. Absence of airway hyperresponsiveness in CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice. BALB/c wild-type (*first and second bar*), CD28/CTLA4$^{-/-}$ (*third and fourth bar*) and B7-1/B7-2$^{-/-}$ mice (*fifth and sixth bar*) were sensitized to OVA adsorbed to alum and challenged with three 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).

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).

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.

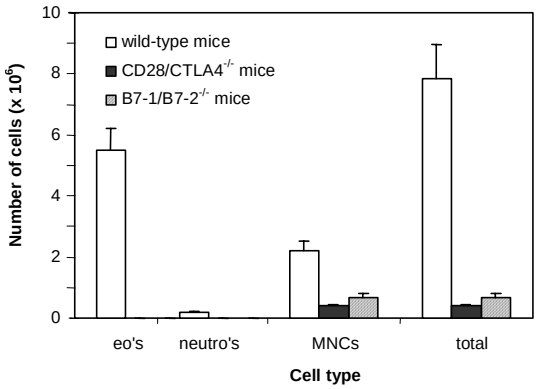

Figure 2. Absence of eosinophils in the BALF from CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice. BALB/c wild-type (*open bars*), CD28/CTLA4$^{-/-}$ (*closed bars*) and B7-1/B7-2$^{-/-}$ mice (*hatched/grey bars*) were sensitized to OVA adsorbed to alum and challenged with three OVA aerosols. At 24 h 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).

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 days. 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, **Fig. 3**) and this amount was reduced compared with cultures from naïve wild-type mice (0.128 ± 0.021 ng/ml, data not shown in **Fig. 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

days 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.

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 **fig. 3**).

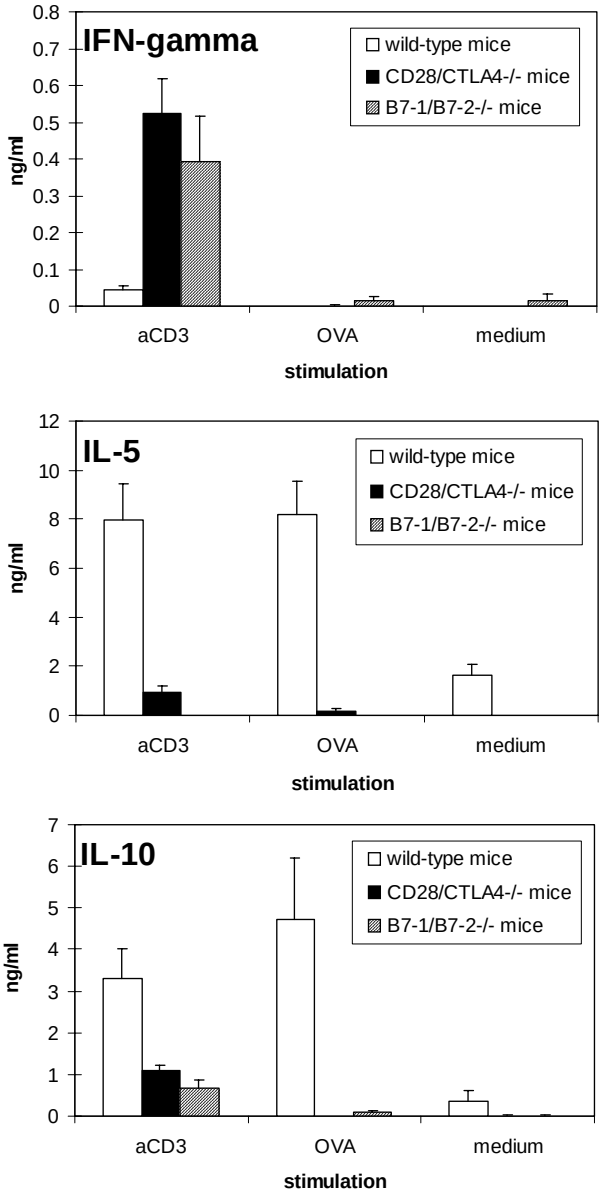

Figure 3. Lung T-cells from CD28/CTLA4$^{-/-}$ and B7-1/B7-2$^{-/-}$ mice are not primed. BALB/c wild-type (*open bars*), CD28/CTLA4$^{-/-}$ (*closed bars*) and B7-1/B7-2$^{-/-}$ mice (*hatched/grey bars*) were sensitized to OVA adsorbed to alum and challenged with three OVA aerosols. At 24 h after the last OVA aerosol challenge, a single-cell suspension of lung tissue was prepared. Lung cells were stimulated for 5 days 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).

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 (13). 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 (14, 15). 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 hyper-responsiveness 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 (16), we failed to induce any of the features of allergic airway sensitization 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 features of allergic airway sensitization 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 (17, 19, 23).

Previously, it has been demonstrated that no features of allergic airway sensitization can be induced in CD28$^{-/-}$ mice (17-19). 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 (20-22). Following the observation that no manifestations of allergic asthma could be induced in CD28$^{-/-}$ mice, two research groups administered CTLA4-Ig to these mice (17, 19) to determine whether the absence of these manifestations 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 (17), whereas treatment of CD28$^{-/-}$ mice with CTLA4-Ig restored lymphocyte but not eosinophil recruitment to the airways in the second study (19).

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 (26) 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 (17, 18) and lymphocytes obtained from the lungs, spleen and lymph nodes respond to antigenic re-stimulation *in vitro* (17-19). 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 immunisation 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 (27). It should be noted, that airway hyperresponsiveness *in vivo* and airway eosinophilia can be induced in mice in the absence of systemic IgE (28) but has been shown to be critically dependent on CD4^{+} cells (29-32). 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*. However, this study cannot exclude the possibility that after initial T-cell activation in the presence of CD28, the supposed B7-1/B7-2 receptor is involved in a later phase of OVA-specific T-cell activation.

The discrepancy of residual T-cell activation in CD28$^{-/-}$ mice (17-19) vs. 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 (17) and Lambrecht *et al.* passively sensitized naïve CD28$^{-/-}$ mice with OVA-pulsed myeloid dendritic cells (18).

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 manifestations of asthma 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 (18). 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 (13). These findings are consistent with the importance of CD28 for Th2 differentiation (23). 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 (13, 15, 22, 33) - 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 (34). 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).

With the caution that a single strain of mice and a single protocol for the induction of features of allergic airway sensitization has been used, the putative B7-1/B7-2 receptor apparently 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 vs. 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 inducible T-cell co-stimulator (ICOS) (35-37). 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-deficient mice are still capable of raising a number of *in vivo* immune responses, including cardiac allograft rejection (13, 15, 22, 33), induction of allogeneic graft-versus-host disease (38), generation of cytotoxic T-cells (39) and certain delayed-type hypersensitivity responses (40), the present study provides further evidence for the critical role of CD28 signaling in the development of Th2-dominated allergic inflammatory responses (17, 19, 23). Moreover, as anticipated by the data on the failure to induce features of allergic airway sensitization in B7-1/B7-2$^{-/-}$ mice (Ref. 16 and this study), T-cell co-stimulatory receptors that interact with ligands other 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.

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References

1. Maddox, L. and D. A. Schwartz. 2002. The pathophysiology of asthma. *Annu.Rev.Med*. 53: 477-498.
2. Robinson, D. S., Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, A. M. Bentley, C. Corrigan, S. R. Durham and A. B. Kay. 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N.Engl. J.Med*. 326: 298-304.
3. Bretscher, P. and M. Cohn. 1970. A theory of self-nonself discrimination. *Science* 169: 1042-1049.
4. Lafferty, K. J. and A. J. Cunningham. 1975. A new analysis of allogeneic interactions. *Aust.J.Exp.Biol.Med.Sci.* 53: 27-42.
5. Baxter, A.G. and P. D. Hodgkin. 2002. Activation rules: the two-signal theories of immune activation. *Nat.Rev.Immunol.* 2: 439-446.
6. Lenschow, D. J., T. L. Walunas and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu.Rev.Immunol.* 14: 233-258.
7. Krinzman, S.J., G.T. De Sanctis, M. Cernadas, D. Mark, Y. Wang, J. Listman, L. Kobzik, C. Donovan, K. Nassr, I. Katona, D. C. Christiani, D. L. Perkins and P. W. Finn. 1996. Inhibition of T cell costimulation abrogates airway hyper-responsiveness in a murine model. *J.Clin.Invest.* 98: 2693-2699.
8. Keane-Myers, A., W. C. Gause, P. S. Linsley, S. J. Chen and M. Wills-Karp. 1997. B7-CD28/CTLA-4 costimulatory pathways are required for the development of T helper cell 2-mediated allergic airway responses to inhaled antigens. *J.Immunol.* 158: 2042-2049.
9. Tsuyuki, S., J. Tsuyuki, K. Einsle, M. Kopf and A. J. Coyle. 1997. Costimulation through B7-2 (CD86) is required for the induction of a lung mucosal T helper cell 2 (TH2) immune response and altered airway responsiveness. *J.Exp.Med.* 185: 1671-1679.
10. Van Oosterhout, A. J., C. L. Hofstra, R. Shields, B. Chan, I. van Ark, P. M. Jardieu, and F. P. Nijkamp. 1997. Murine CTLA4-IgG treatment inhibits airway eosinophilia and hyper-responsiveness and attenuates IgE upregulation in a murine model of allergic asthma. *Am.J.Respir.Cell.Mol. Biol.* 17: 386-392.
11. Padrid, P. A., M. Mathur, X. Li, K. Herrmann, Y. Qin, A. Cattamanchi, J. Weinstock, D. Elliott, A. I. Sperling, and J. A. Bluestone. 1998. CTLA4Ig inhibits airway eosinophilia and hyper-responsiveness by regulating the development of Th1/Th2 subsets in a murine model of asthma. *Am.J. Respir.Cell.Mol.Biol.* 18: 453-462.
12. Deurloo, D.T., B. C. van Esch, C. L. Hofstra, F. P. Nijkamp, and A. J. van Oosterhout 2001. CTLA4-IgG reverses asthma manifestations in a mild but not in a more "severe" ongoing murine model. *Am.J.Respir.Cell.Mol. Biol.* 25: 751-760.

13. Mandelbrot, D. A., M. A. Oosterwegel, K. Shimizu, A. Yamada, G. J. Freeman, R. N. Mitchell, M. H. Sayegh, and A. H. Sharpe. 2001. B7-dependent T-cell costimulation in mice lacking CD28 and CTLA4. *J.Clin.Invest.* 107: 881-887.
14. Mandelbrot, D. A., Y. Furukawa, A. J. McAdam, S. I. Alexander, P. Libby, R. N. Mitchell, and A. H. Sharpe. 1999. Expression of B7 molecules in recipient, not donor, mice determines the survival of cardiac allografts. *J.Immunol.* 163: 3753-3757.
15. Szot, G.L., P. Zhou, A. H. Sharpe, G. He, O. Kim, K. A. Newell, J. A. Bluestone, and J. R. Thistlethwaite Jr. 2000. Absence of host B7 expression is sufficient for long-term murine vascularized heart allograft survival. *Transplantation* 69: 904-909.
16. Mark, D. A., C. E. Donovan, G. T. De Sanctis, H. Z. He, M. Cernadas, L. Kobzik, D. L. Perkins, A. Sharpe and P. W. Finn. 2000. B7-1 (CD80) and B7-2 (CD86) have complementary roles in mediating allergic pulmonary inflammation and airway hyperresponsiveness. A*m.J.Respir.Cell.Mol. Biol.* 22: 265-271.
17. Mathur, M., K. Herrmann, Y. Qin, F. Gulmen, X. Li, R. Krimins, J. Weinstock, D. Elliott, J. A. Bluestone and P. Padrid. 1999. CD28 interactions with either CD80 or CD86 are sufficient to induce allergic airway inflammation in mice. *Am.J.Respir. Cell.Mol.Biol.* 21: 498-509.
18. Lambrecht, B. N., M. de Veerman, A. J. Coyle, J. C. Gutierrez-Ramos, K. Thielemans and R. A. Pauwels. 2000. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J.Clin.Invest.* 106: 551-559.
19. Burr, J. S., S. L. Kimzey, D. R. Randolph and J. M. Green. 2001. CD28 and CTLA4 coordinately regulate airway inflammatory cell recruitment and T-helper cell differentiation after inhaled allergen. *Am.J. Respir.Cell.Mol. Biol.* 24: 563-568.
20. Fallarino, F., P.E. Fields, and T.F. Gajewski. 1998. B7-1 engagement of cytotoxic T lymphocyte antigen 4 inhibits T cell activation in the absence of CD28. *J.Exp.Med.* 188: 205-210.
21. Lee, K.M., E. Chuang, M. Griffin, R. Khattri, D. K. Hong, W. Zhang, D. Straus, L. E. Samelson, C. B. Thompson and J. A. Bluestone. 1998. Molecular basis of T cell inactivation by CTLA-4. *Science* 282: 2263-2266.
22. Lin, H., J. C. Rathmell, G. S. Gray, C. B. Thompson, J. M. Leiden and M. L. Alegre. 1998. Cytotoxic T lymphocyte antigen 4 (CTLA4) blockade accelerates the acute rejection of cardiac allografts in CD28-deficient mice: CTLA4 can function independently of CD28. *J.Exp.Med.* 188: 199-204.
23. Rulifson, I. C., A. I. Sperling, P. E. Fields, F. W. Fitch and J. A. Bluestone. 1997. CD28 costimulation promotes the production of Th2 cytokines. *J.Immunol.* 158: 658-665.
24. Borriello, F., M. P. Sethna, S. D. Boyd, A. N. Schweitzer, E. A. Tivol, D. Jacoby, T. B. Strom, E. M. Simpson, G. J. Freeman and A. H. Sharpe. 1997. B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. *Immunity* 6: 303-313.
25. Hamelmann, E., J. Schwarze, K. Takeda, A. Oshiba, G. L. Larsen, C. G. Irvin, and

E.W. Gelfand. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am.J. Respir.Crit.Care Med.* 156: 766-775.

26. Judge, T. A., Z. Wu, X. G. Zheng, A. H. Sharpe, M. H. Sayegh, and L. A. Turka. 1999. The role of CD80, CD86, and CTLA4 in alloimmune responses and the induction of long-term allograft survival. *J.Immunol.* 162: 1947-1951.
27. Coffman, R. L., J. Ohara, M. W. Bond J., Carty, A. Zlotnik, and W. E. Paul. 1986. B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J.Immunol.* 136: 4538-4541.
28. Mehlhop, P. D., M. van de Rijn, A. B. Goldberg, J. P. Brewer, V. P. Kurup, T. R. Martin and H. C. Oettgen. 1997. Allergen-induced bronchial hyper-reactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. *Proc.Natl.Acad.Sci.USA* 94: 1344-1349.
29. Gavett, S. H., X. Chen, F. Finkelman, and M. Wills-Karp. 1994. Depletion of murine CD4^{+} T lymphocytes prevents antigen-induced airway hyper-reactivity and pulmonary eosinophilia. *Am.J.Respir.Cell.Mol.Biol.* 10: 587-593.
30. Hofstra, C. L., I. Van Ark, H. F. Savelkoul, W. W.Cruikshank, F. P. Nijkamp and A. J. Van Oosterhout. 1998. Vbeta8+ T lymphocytes are essential in the regulation of airway hyper-responsiveness and broncho-alveolar eosinophilia but not in allergen-specific IgE in a murine model of allergic asthma. *Clin.Exp.Allergy* 28: 1571-1580.
31. Hogan, S.P., K. I. Matthaei, J. M. Young, A. Koskinen, I. G. Young and P. S. Foster. 1998. A novel T cell-regulated mechanism modulating allergen-induced airways hyper-reactivity in BALB/c mice independently of IL-4 and IL-5. *J.Immunol.* 161: 1501-1509.
32. Wilder, J. A., D. D. Collie, D. E. Bice, Y. Tesfaigzi, C. R. Lyons and M. F. Lipscomb. 2001. Ovalbumin aerosols induce airway hyperreactivity in naïve DO11.10 T cell receptor transgenic mice without pulmonary eosinophilia or OVA-specific antibody. *J.Leukoc.Biol.* 69: 538-547.
33. Pearson, T. C., D. Z. Alexander, M. Corbascio, R. Hendrix, S. C. Ritchie, P. S. Linsley, D. Faherty, and C.P. Larsen. 1997. Analysis of the B7 costimulatory pathway in allograft rejection. *Transplantation* 63: 1463-1469.
34. Stevens, T.L., A. Bossie, V. M. Sanders, R. Fernandez-Botran, R. L. Coffman, T. R. Mosmann, and E. S. Vitetta. 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* 334: 255-258.
35. Bonecchi, R., G. Bianchi, P. P. Bordignon, D. D'Ambrosio, R. Lang, A. Borsatti, S. Sozzani, P. Allavena, P. A. Gray, A. Mantovani and F. Sinigaglia. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J.Exp.Med.* 187: 129-134.
36. Coyle, A. J., S. Lehar, C. Lloyd, J. Tian, T. Delaney, S. Manning, T. Nguyen, T. Burwell, H. Schneider, J. A. Gonzalo, M. Gosselin, L. R. Owen, C. E. Rudd, and J. C. Gutierrez-Ramos. 2000. The CD28-related molecule ICOS is required for effective T cell- dependent immune

responses. *Immunity* 13: 95-105.

37. McAdam, A. J., T. T. Chang, A. E. Lumelsky, E. A. Greenfield, V. A. Boussiotis, J. S. Duke-Cohan, T. Chernova, N. Malenkovich, C. Jabs, V. K. Kuchroo, V. Ling, M. Collins, A. H. Sharpe and G. J. Freeman. 2000. Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of $CD4^+$ T cells. *J.Immunol.* 165: 5035-5040.
38. Speiser, D. E., M. F. Bachmann, A. Shahinian, T. W. Mak and P. S. Ohashi. 1997. Acute graft-versus-host disease without costimulation via CD28. *Transplantation* 63: 1042-1044.
39. Wen, T., K. Kono, A. Shahinian, R. Kiessling, T. W. Mak, and G. Klein. 1997. CD28 is not required for rejection of unmanipulated syngeneic and autologous tumors. *Eur.J. Immunol.* 27: 1988-1993.
40. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261: 609-612.

Chapter 7

General discussion

Summarizing conclusions

Costimulatory molecules act in conjunction to the TCR for regulation of T cell activation. Members of the B7-family of costimulatory molecules are mandatory for a good balance between activation and inhibition of a T cell response. CD28 and ICOS are the two most important positive costimulators of this family. Since ICOS is homologous to CD28, we questioned which functions are similar and distinct between ICOS and CD28 (described in **chapter 1**). In this thesis, we have demonstrated that ICOS resembles CD28 functioning in terms of induction of proliferation (**chapter 3**), survival and differentiation (**chapter 4**) and T helper effector function (**chapter 5**) of $CD4^+$ T cells. Next to these similarities in function, unique functions can be ascribed to either CD28 or ICOS.

ICOS and CD28 induction of proliferation in $CD4^+$ T cells

In **chapter 3** it was demonstrated that next to CD28, ICOS-mediated costimulation drives $CD4^+$ T cell expansion *in vivo* in the absence of CTLA-4. Expansion of the T cell pool is the net result of proliferation and survival of T cells. We showed *in vitro* that murine T cells proliferate in response to ICOS-induced costimulation, but only in the presence of supplemental IL-2. IL-2 supports growth of TCR activated T cells by promoting cell cycle entry and induces survival of T cells (1). In murine $CD4^+$ T cells, supplemental IL-2 or CD28 triggering elevates the expression of ICOS, which may be prerequisite for ICOS induced T cell proliferation. Since IL-2 is a cytokine, which is highly induced after CD28-mediated costimulation one hypothesis is that CD28-mediated signaling is required for ICOS function. Arguing against this hypothesis is the fact that $CD28^{-/-}$ T cells can respond to ICOS-mediated costimulation, indicating that ICOS functions independently from CD28 (unpublished results) (2). In contrast to murine T cells, peripheral human T cells are readily activated by ICOS, resulting in T cell proliferation (**chapter 4**) while no additional IL-2 is required. This was not due to the presence of Ag-experienced T cells, since naïve $CD45RA^+$ $CD4^+$ T cells could also be co-stimulated via ICOS. Despite differences between ICOS induced proliferation in murine and human T cells in conjunction with the TCR, αCD28 was more effective in the induction of proliferation compared to αICOS in both cell types (unpublished data). The greater potential of CD28 to induce T cell proliferation compared to ICOS is supported by a more severe reduced primary T cell proliferation in T cells lacking CD28 compared to $ICOS^{-/-}$ T cells (3), suggesting that a difference in induction of proliferation *in vitro* with Abs is unlikely to result from a difference in agonistic potential between the two Abs.

Current models describe that activation of naïve T cells occurs when a certain threshold is overcome by the combined signaling through the TCR and costimulatory molecules. ICOS is present at low levels or readily (within hours) upregulated after TCR engagement, so, why is ICOS less sufficient in T cell activation of naïve T cells compared to CD28? There are two possible explanations: either the strength of signal generated through ICOS is not sufficient to overcome signaling threshold or ICOS-mediated signals are qualitatively not sufficient to initiate T cell

activation in naïve T cells, or both, as discussed next.

ICOS- and CD28-mediated costimulation in naïve CD4+ T cells: threshold difference?

Co-ligation of CD28 can reduced the number of TCRs that must be ligated for a response, thereby lowering the activation threshold (4). Similarly, the overall strength of signals generated through ICOS to overcome the TCR threshold could be dependent on the number of molecules of ICOS present on the T cell surface and the availability of the B7RP-1 ligand. Mice transgenic for soluble B7RP-1-Ig *in vivo* resulted in a T cell proliferative phenotype, as agonistic αICOS Ab treatment does in wild type mice (2) (unpublished observation). Apparently, the low ICOS expression is sufficient to drive murine T cell expansion when enough B7RP-1 is provided *in vivo*; while *in vitro* these cells were refractory to ICOS-induced costimulation. The difference might be explained by the presence of APCs expressing a variety of other costimulatory molecules *in vivo*, while *in vitro* purified $CD4^+$ T cells were used. B7/CD28 interaction did not contribute to the *in vivo* T cell expansion, since treatment of $CD28^{-/-}$-$CTLA\text{-}4^{-/-}$ mice with agonistic ICOS agents also caused lymphoproliferation (unpublished result). Notably, in contrast to agonistic ICOS agents, continued CD28 stimulation did not induce T cell proliferation *in vivo*, most probably due to the maintenance of tolerance induced by CTLA-4 (5,6). In summary, in conjunction to a TCR signal, ICOS is capable of induction of proliferation in T cells per se, once strong enough ligation is provided, which could overcome a certain TCR threshold for activation. Whether the expression of ICOS and availability of B7RP-1 under physiological conditions *in vivo* allow sufficient activation of naïve T cell remains to be seen.

ICOS- and CD28-mediated costimulation in naïve CD4+ T cells: signaling difference?

Next to difference in expression on the surface of naïve T cells and ligand availability there is evidence that both ICOS and CD28 signal in different ways. First, the ICOS intracellular tail lacks motifs that are present in CD28 and mediate the early T cell processes. For instance, Lck is recruited to the proximal proline-rich region of CD28 intracellular tail respectively, which was shown to enhance early events of T cell activation (7). In addition, ICOS intracellular tail does not recruit Grb-2, important for the production of IL-2 a cytokine important for clonal expansion (8). Second, signaling pathways downstream CD28 and ICOS are different. In conjunction to the TCR signals, downstream signaling pathways activated by CD28 include enhanced activation of PI3K and subsequently PKB (protein kinase B) and PDK1 (phosphoinositide-dependent kinase 1) activation, inhibition of GSK3 (glycogen synthase kinase-3) and increased Ca^{2+} influx leading to calcineurin activation (reviewed in (9, 10). Inhibition of GSK3, a NFAT nuclear export kinase, and increased calcineurin activity, a NFAT phosphatase, lead to nuclear transport of NFAT transcription factor (nuclear factor of activated cells). Simultaneously, JNK is activated via the Grb-2-sos pathway and induces formation of the AP-1 transcription

complex (11). Joined nuclear localization of AP-1 and NFAT lead to activation of target genes, such as IL-2. ICOS signaling enhances activation of PI3K and NFAT nuclear localization, but does not lead to JNK activation (12, 13). The absence of proper AP-1 complex formation after ICOS-mediated costimulation may be crucial difference between CD28 and ICOS in activation of T cells.

We have performed experiments to compare ICOS and CD28 functioning in $CD4^+$ T cell activation when both molecules are expressed simultaneously on the T cell surface. Therefore, $CD28^{-/-}$ T cells were reconstituted with either CD28 wild type or CD28 (extracellular)-ICOS (intracellular tail) chimeric molecules and activated with APCs with Ag. In this experimental model, CD28-ICOS chimera did not restore T cell proliferation nor survival deficiency in $CD28^{-/-}$ T cells, while CD28 wild type molecules did (unpublished results). In contrast to potent enhancement of TCR/ICOS-induced proliferation in wild type murine T cells by exogenous IL-2, the addition of exogenous IL-2 could not mediate induction of proliferation via CD28-ICOS chimeric molecules in $CD28^{-/-}$ T cells stimulated with APCs and Ag. Apparently, under these experimental conditions, ICOS cannot equally regulate T cell activation compared to CD28 in CD28-deficient T cells. This could be due to disability of ICOS to reverse a state of unresponsiveness, so called anergic state, of the $CD28^{-/-}$ T cells (14). A state of anergy as observed in $CD28^{-/-}$ T cells could be caused by a disbalance between NFAT and AP-1 activation in the nucleus of the T cell (15). TCR signaling alone leads to NFAT activation, but is unable to mount sufficient AP-1 translocation in the absence of CD28-mediated costimulation. In the presence of a TCR signal it is possible that ICOS, in contrast to CD28 cannot reverse the anergic state of $CD28^{-/-}$ T cells, due to the lack of sufficient AP-1 activation after ICOS-mediated costimulation. If this hypothesis holds true, than simultaneous JNK activation in addition to triggering of TCR and CD28-ICOS chimeric molecules in $CD28^{-/-}$ T cells would supplement the induction of proliferation in these T cells.

In addition, the inability of CD28-ICOS chimeric molecules to activate $CD28^{-/-}$ T cells might be due to a difference in induction of survival signals generated by ICOS compared to CD28. However, comparative studies between CD28 and ICOS have revealed that $CD4^+$ T cell viability as well as pro-survival genes were similarly regulated by ICOS and CD28 in $CD4^+$ T cells **(chapters 3 and 4).** Alternatively, other yet unidentified mechanisms are induced by wild type CD28 and not by CD28-ICOS chimera in $CD28^{-/-}$ T cells.

Taken together, differences in downstream signaling pathways of CD28 and ICOS could explain the disability of ICOS to induce activation in naïve murine T cells. CD28 predominates over ICOS with respect to function in initiating naïve $CD4^+$ T cell activation, based on expression and signaling divergence of both molecules, but can readily be supplemented by ICOS for further expansion.

Role of putative CD28 homologue in immune response

Next to a unique role for CD28 in the initiation of T cell activation, both ICOS and CD28 have substantial overlapping

functions in the subsequent immune response. In **chapter 5** it was demonstrated that the absence of either CD28 or ICOS resulted in reduced adjuvant activity of *Neisseria meningitidis* LPS in terms of IgG production. CD28 and ICOS cooperatively accounted for the entire IgG response against *N. meningitides*, since no IgG could be detected in mice defective for both pathways. It should be noted that these studies were performed in B7.1/2-ICOS triple deficient mice, which also lack signaling via the third unidentified B7.1/2 receptor. This CD28-like molecule has been described to be important in chronic allograft rejection, but does not contribute to a Th2 driven allergic airway response, as described in **chapter 6**. Other groups showed that CD28-ICOS double deficient mice lack detectable IgG production after immunization, which rules out a potential role for the putative receptor in a T cell dependent B cell response (3). To ascertain the lack of involvement of this putative receptor in this model immunized $CD28^{-/-}$ mice should be treated with CTLA-4-Ig, which would not further reduce the IgG response against *N. meningitidis.*

Contribution of ICOS-B7RP-1 to LPS adjuvant activity

Adjuvant activity of LPS is ascribed to the potent induction of costimulatory molecules on professional APCs that initiate the adaptive response. Both CD28 and ICOS contributed to the adjuvant activity of *N. meningitidis* LPS, as observed by a reduction in serum IgG in $CD28^{-/-}$ and $ICOS^{-/-}$ mice after immunization in the presence of *lpxL1* LPS. Notably, the adjuvant activity of mutant *lpxL1* LPS compared to wild type H44/76 LPS was more dependent on ICOS-mediated costimulation. One explanation for the ICOS dependent adjuvant activity is the availability of the ligand, as described in **chapter 5.** B7RP-1 expression is high on immature DCs and maintains relatively high after stimulation in the presence of *lpxL1* LPS, while H44/76 LPS causes more severe downregulation of B7RP-1. However, H44/76 LPS is a more potent inducer of B7.1/2 expression on DCs than *lpxL1* LPS, which did not result in a more CD28 dependent response *in vivo.* This suggest either that the low levels of B7 are sufficient to allow optimal CD28-driven immune responses or that alternative mechanisms than ligand availability might influence the contribution of CD28 or ICOS to the adjuvant activity. For instance, members of other costimulatory families might contribute to the IgG response after immunization in the presence of strong adjuvants.

TLRs on other cells

In addition to DC activation, LPS containing OMCs may directly influence function of other cells contributing to IgG production, such as B and T cells. Studying the requirement for TLRs revealed the use of TLR2 and TLR4 by OMCs (L. Steeghs, personal communication). While LPS-deficient OMC signaled via TLR2, TLR4 was necessary for H44/76 LPS and *lpxL1* LPS induced DC activation. Next to expression on cells of the innate immune systems, TLR2 and TLR4 have been found to be expressed on T cells where TLR2 ligation could cause costimulation of human T cells together with the TCR (16, 17). Although the functional relevance of expression of TLR4 on conventional $CD4^+$ T cells is controversial, the effect of OMCs

containing LPS or *lpxL1* LPS directly on activation of T cells should be considered. In addition, recently a down-regulating function of TLRs on Treg has been described next to the well-known activating function of triggering of TLRs on other immune cells. Naturally occurring $CD4^+$ regulatory T cells have been described to contain TLR2 and TLR4 on the surface (18, 19). Stimulation through these receptors could induce proliferation and suppressive activity of Treg, which protects the host from self-destructive inflammation. Whether LPS and *lpxL1* LPS differentially influence activation of conventional and regulatory T cells and perhaps expression of ICOS on these cells remains to be determined.

Alternatively, LPS could act directly on TLRs expressed on B cells. TLR ligation on B cells was recently shown to be mandatory for a T cell dependent immune response, which resulted in enhanced IgG production (20). Although direct TLR ligation in B cells likely contributes to the eventual IgG response, fact remains that *in vivo* IgG response is dependent on the joined requirement of CD28 and ICOS. TLR ligation on B cells could result in differential display of B7 counterparts of CD28 and ICOS, which in turn influences the dependency on either pathway. Preliminary results show that both LPS and *lpxL1* LPS regulated expression of B7RP-1 and B7.2/1 on B cells. The latter were upregulated, while B7RP-1 were not downregulated as much as observed on DCs. A difference between H44/76 LPS and *lpxL1* LPS directly on B and T cell activation remains to be determined.

ICOS on regulatory T cells

Another puzzling question is the high expression of B7RP-1 on immature murine DCs. While B7.1/2 are low on immature DCs, B7RP-1 is highly expressed on these cells and is even downmodulated upon maturation with LPS (**chapter 5**). Low B7.1/2 expression on APC has been demonstrated to function for maintance and activation of Treg (21). Similarly, high B7RP-1 on immature DCs could provide maintenance of Treg activity through ICOS. In agreement with this, ICOS is highly expressed on Treg and blocking ICOS abrogated the suppressive function of these cells (22-24). Maintance of Treg could provide an additional function for ICOS and downmodulation of B7RP-1 may in turn release suppressive function of Treg and allow T cell activation.

The absence of functional Treg could contribute to the phenotype of CTLA-$4^{-/-}$ mice described in **chapter 3**, since CTLA-4 is important for the suppressive function of Treg (25, 26). ICOS contributes to Treg effector function, so antagonistic αICOS Ab may reduce the suppressive function of Treg. Given the fact that inhibiting ICOS reduced T cell numbers rather than exacerbating it suggests that in this model the activatory function in conventional cells outweighs the regulatory function in Treg. Similarly, $ICOS^{-/-}$ and B7RP-$1^{-/-}$ mice do not display enhanced auto-immunity reactivity, although one report describes the accelerated onset of EAE disease when ICOS was blocked (27). Nevertheless, the contribution of ICOS and CD28 to Treg function cannot be excluded to contribute to an *in vivo* T cell response and the outcome of stimulation via these two receptors might

be a mix between activation of conventional $CD4^+$ T cells and inhibition of Treg.

Unknown ICOS: B7RP-1-mediated functions

One yet unidentified result of B7RP-1: ICOS ligation is the effect on the APC by this interaction as has also been described for B7.1/2 ligation by CD28. Although the B7.1/2 tail is comprised of only 25 AA, it does appear to signal into the DC (28). ICOS-Ig could, similarly to CD28-Ig cause signal transduction in the DC. No obvious motifs nor similarities are apparent between B7RP-1 and B7.1/2 intracellular tails (2, 29). The reciprocal signaling via B7RP-1 could alter expression of additional costimulatory molecules, such as CD40, or increased cytokines necessary for T cell activation or differentiation.

Concluding remarks

In summary, CD28- and ICOS-mediated functions in $CD4^+$ T cells show similarities and differences. Uniquely described for CD28 are IL-2 production, high expression on naïve cells, negative feedback by counterpart CTLA-4 and the role in Treg development in the thymus. Similarities include induction of T cell expansion, T helper cell differentiation, maintenance and activation of Treg and T:B cell help. The question remains: why are there two costimulatory molecules that function in many similar ways? So why is B7RP-1:ICOS necessary in immune response in addition to B7.1/2: CD28? Although CD28-induced functions seem to overlap ICOS function completely, defects in $ICOS^{-/-}$ mice demonstrate that there are ICOS-mediated functions that cannot be compensated by the presence of CD28. More research will shed light on yet unknown factors that could ascribe unique functions to ICOS, which are additional to CD28-mediated response.

References

1. Benczik, M. and S. L. Gaffen. 2004. The interleukin (IL)-2 family cytokines: survival and proliferation signaling pathways in T lymphocytes. *Immunol.Invest.* 33:109-142.
2. Yoshinaga, S. K., J. S. Whoriskey, S. D. Khare, U. Sarmiento, J. Guo, T. Horan, G. Shih, M. Zhang, M. A. Coccia, T. Kohno, A. Tafuri-Bladt, D. Brankow, P. Campbell, D. Chang, L. Chiu, T. Dai, G. Duncan, G. S. Elliott, A. Hui, S. M. McCabe, S. Scully, A. Shahinian, C. L. Shaklee, G. Van, T. W. Mak, and G. Senaldi. 1999. T-cell co-stimulation through B7RP-1 and ICOS. *Nature* 402:827-832.
3. Suh, W. K., A. Tafuri, N. N. Berg-Brown, A. Shahinian, S. Plyte, G. S. Duncan, H. Okada, A. Wakeham, B. Odermatt, P. S. Ohashi, and T. W. Mak. 2004. The inducible costimulator plays the major costimulatory role in humoral immune responses in the absence of CD28. *J.Immunol.* 172:5917-5923.
4. Bachmann, M. F., K. McKall-Faienza, R. Schmits, D. Bouchard, J. Beach, D. E. Speiser, T. W. Mak, and P. S. Ohashi. 1997. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity* 7:549-557.
5. Mandelbrot, D. A., A. J. McAdam, and A. H. Sharpe. 1999. B7-1 or B7-2 is required to produce the lympho-proliferative phenotype in mice lacking cytotoxic T lymphocyte-associated

antigen 4 (CTLA-4). *J.Exp.Med.* 189:435-440.

6. Van Parijs, L., M. P. Sethna, A. N. Schweitzer, F. Borriello, A. H. Sharpe, and A. K. Abbas. 1997. Functional consequences of dysregulated B7-1 (CD80) and B7-2 (CD86) expression in B or T lymphocytes of transgenic mice. *J.Immunol.* 159:5336-5344.
7. Raab, M., Y. C. Cai, S. C. Bunnell, S. D. Heyeck, L. J. Berg, and C. E. Rudd. 1995. p56Lck and p59Fyn regulate CD28 binding to phosphatidylinositol 3-kinase, growth factor receptor-bound protein GRB-2, and T cell-specific protein-tyrosine kinase ITK: implications for T-cell costimulation. *Proc.Natl.Acad.Sci.U.S.A.* 92:8891-8895.
8. Harada, Y., D. Ohgai, R. Watanabe, K. Okano, O. Koiwai, K. Tanabe, H. Toma, A. Altman, and R. Abe. 2003. A single amino acid alteration in cytoplasmic domain determines IL-2 promoter activation by ligation of CD28 but not inducible costimulator (ICOS). *J.Exp.Med.* 197:257-262.
9. Rudd, C. E. and H. Schneider. 2003. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. *Nat.Rev.Immunol.* 3:544-556.
10. Diehn, M., A. A. Alizadeh, O. J. Rando, C. L. Liu, K. Stankunas, D. Botstein, G. R. Crabtree, and P. O. Brown. 2002. Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation. *Proc.Natl.Acad.Sci.U.S.A.* 99:11796-11801.
11. Su, B., E. Jacinto, M. Hibi, T. Kallunki, M. Karin, and Y. Ben Neriah. 1994. JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* 77:727-736.
12. Nurieva, R. I., J. Duong, H. Kishikawa, U. Dianzani, J. M. Rojo, I. Ho, R. A. Flavell, and C. Dong. 2003. Transcriptional regulation of th2 differentiation by inducible costimulator. *Immunity* 18:801-811.
13. Arimura, Y., H. Kato, U. Dianzani, T. Okamoto, S. Kamekura, D. Buonfiglio, T. Miyoshi-Akiyama, T. Uchiyama, and J. Yagi. 2002. A co-stimulatory molecule on activated T cells, H4/ICOS, delivers specific signals in T(h) cells and regulates their responses. *Int.Immunol.* 14:555-566.
14. Schwartz, R. H. 2003. T cell anergy. *Annu.Rev.Immunol.* 21:305-334.
15. Appleman, L. J. and V. A. Boussiotis. 2003. T cell anergy and costimulation. *Immunol.Rev.* 192:161-180.
16. Matsuguchi, T., K. Takagi, T. Musikacharoen, and Y. Yoshikai. 2000. Gene expressions of lipopolysaccharide receptors, toll-like receptors 2 and 4, are differently regulated in mouse T lymphocytes. *Blood* 95:1378-1385.
17. Komai-Koma, M., L. Jones, G. S. Ogg, D. Xu, and F. Y. Liew. 2004. TLR2 is expressed on activated T cells as a costimulatory receptor. *Proc.Natl.Acad. Sci.U.S.A.* 101:3029-3034.
18. Gelman, A. E., J. Zhang, Y. Choi, and L. A. Turka. 2004. Toll-like receptor ligands directly promote activated $CD4^+$ T cell survival. *J.Immunol.* 172:6065-6073.
19. Caramalho, I., T. Lopes-Carvalho, D. Ostler, S. Zelenay, M. Haury, and J. Demengeot. 2003. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J.Exp.Med.* 197:403-411.
20. Pasare, C. and R. Medzhitov. 2005. Control of B-cell responses by Toll-like

receptors. *Nature* 438:364-368.

21. Lohr, J., B. Knoechel, S. Jiang, A. H. Sharpe, and A. K. Abbas. 2003. The inhibitory function of B7 costimulators in T cell responses to foreign and self-antigens. *Nat.Immunol.* 4:664-669.
22. Akbari, O., G. J. Freeman, E. H. Meyer, E. A. Greenfield, T. T. Chang, A. H. Sharpe, G. Berry, R. H. DeKruyff, and D. T. Umetsu. 2002. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyper-reactivity. *Nat.Med.* 8:1024-1032.
23. Lohning, M., A. Hutloff, T. Kallinich, H. W. Mages, K. Bonhagen, A. Radbruch, E. Hamelmann, and R. A. Kroczek. 2003. Expression of ICOS in vivo defines $CD4^+$ effector T cells with high inflammatory potential and a strong bias for secretion of interleukin 10. *J.Exp.Med.* 197:181-193.
24. Herman, A. E., G. J. Freeman, D. Mathis, and C. Benoist. 2004. $CD4^+CD25^+$ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. *J.Exp.Med.* 199:1479-1489.
25. Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD25^+CD4^+$ regulatory cells that control intestinal inflammation. *J.Exp.Med.* 192:295-302.
26. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by $CD25^+CD4^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J.Exp.Med.* 192:303-310.
27. Rottman, J. B., T. Smith, J. R. Tonra, K. Ganley, T. Bloom, R. Silva, B. Pierce, J. C. Gutierrez-Ramos, E. Ozkaynak, and A. J. Coyle. 2001. The costimulatory molecule ICOS plays an important role in the immunopathogenesis of EAE. *Nat.Immunol.* 2:605-611.
28. Orabona, C., U. Grohmann, M. L. Belladonna, F. Fallarino, C. Vacca, R. Bianchi, S. Bozza, C. Volpi, B. L. Salomon, M. C. Fioretti, L. Romani, and P. Puccetti. 2004. CD28 induces immunostimulatory signals in dendritic cells via CD80 and CD86. *Nat.Immunol.* 5:1134-1142.
29. Wang, S., G. Zhu, A. I. Chapoval, H. Dong, K. Tamada, J. Ni, and L. Chen. 2000. Costimulation of T cells by B7-H2, a B7-like molecule that binds ICOS. *Blood* 96:2808-2813.

Samenvatting

Het immuunsysteem

Het menselijke lichaam bevat een uniek systeem, het immuunsysteem, wat ons beschermt tegen ongewenste en schadelijk indringers (ook wel lichaamsvreemde substanties of pathogenen genoemd) zoals bacteriën en virussen. Het immuunsysteem herkent en vernietigt deze pathogenen en vormt zelfs een geheugen zodat het bij een volgende keer dat hetzelfde pathogeen het lichaam binnendringt sneller en efficiënter kan reageren. Om een goede afweer reactie (immuun respons) te kunnen krijgen bestaat het immuunsysteem uit een heel scala aan zogenaamde witte bloedcellen. Verschillende witte bloedcellen, met elk hun specifieke functie, werken samen om de pathogenen onschadelijk te maken en een geheugen te vormen om ook in de toekomst daartegen beschermt te zijn.

T cellen

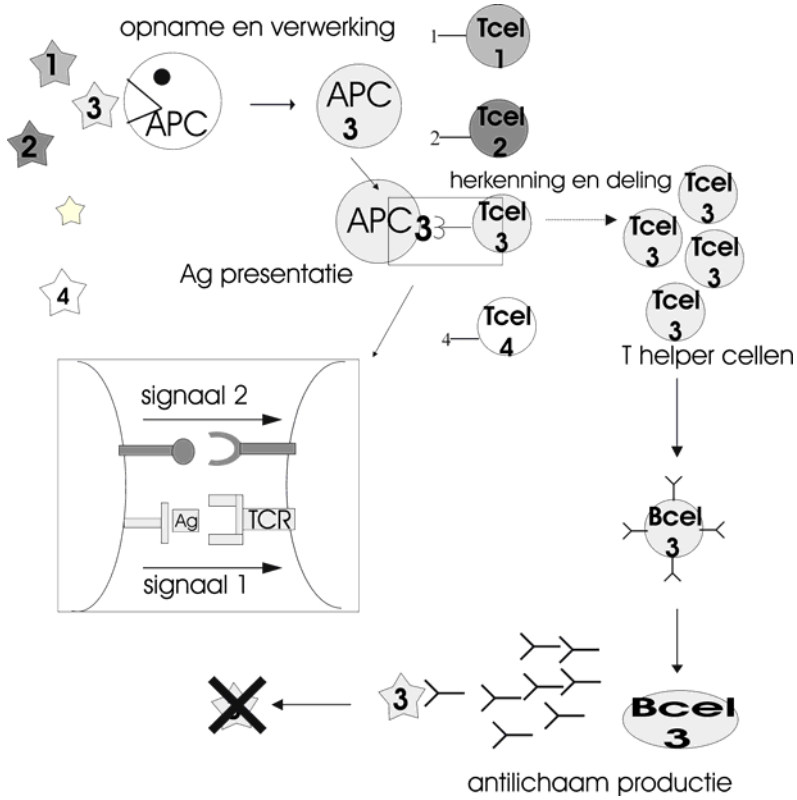
Een groot gedeelte van de witte bloed cellen van het immuunsysteem zijn de zogenaamde T cellen. T cellen zijn gespecialiseerd in het onschadelijk maken van lichaamsvreemde substanties, wat kan gebeuren op een directe (door zelf stoffen uit te scheiden die dodelijk zijn) of indirecte (door het aansturen van andere cellen) manier. Belangrijk voor de werking van deze cellen is dat ze (1) in actie komen als lichaamsvreemde substanties herkend worden, (2) alleen reageren op voor het lichaam gevaarlijke stoffen en (3) dat de werking van de cellen stopt als de lichaamsvreemde substantie uit het lichaam verdwenen is. Het uitblijven van actie van T cellen kan resulteren in voor het lichaam gevaarlijke chronische infecties. Aan de andere kant, als T cellen reageren op stoffen die in het lichaam zelf voorkomen, kan dat resulteren in een ongewenste immuun reactie tegen lichaamseigen stoffen (auto-immuniteit). Het is daarom van belang dat de T cellen goed gecontroleerd in actie komen. De rol van de zogenaamde antigen presenterende cellen (APC) is hierbij essentieel.

Herkenning van lichaamsvreemde stoffen

De herkenning van een lichaamsvreemde substantie is het begin van een T cel-gemedieerde immuun respons (**figuur 1**). Als een pathogeen het lichaam binnendringt wordt het in eerste instantie herkend en opgegeten door de APCs. Deze cellen bewerken het pathogeen en hakken het in kleine stukjes, de zogenaamde antigenen. Een antigen is een klein deel van een lichaamsvreemde stof dat gebruikt wordt door de cellen van het immuunsysteem als herkenning van het pathogeen. Deze antigenen laat de APC vervolgens aan de T cellen zien met de boodschap: 'substanties die dit antigen hebben zijn gevaarlijk en moeten onschadelijk gemaakt worden'. Moleculen op het oppervlak van de T cel, de T cel receptor (TCR), herkennen dit antigen. Ons lichaam heeft een groot aantal verschillende T cellen die allemaal 'opgeleid' zijn om een specifiek stukje/antigen van een lichaamvreemde stof te herkennen (**Figuur 1**, T cel 1 specifiek voor antigen 1, T cel 2 voor antigen 2, enz). Deze cellen circuleren allemaal in het bloed wachtend op herkenning van dat stukje/antigen waarvoor ze zijn opgeleid (een 'rustende' T cel). Zodra een T cel het antigen herkent waarvoor deze is opgeleid dan krijgt ze tevens extra signalen van de APC om in actie te komen.

T cellen in actie

Naast het feit dat de APC het antigen laat zien aan de voor het antigen specifieke T cel, geeft het ook instructies mee hoe de T cel het beste het pathogeen kan gaan aanvallen. Deze instructies zijn tweeledig: (1) de antigen- specifieke T cel moet zich gaan vermeerderen en (2) hoe deze T cel moet veranderen om het pathogeen zo effectief mogelijk te vernietigen. Het vermeerderen van een T cel zal na herkenning van het antigen gebeuren door celdeling, zodat er meer cellen ontstaan die het pathogeen kunnen gaan aanvallen. Pas nadat de antigen specifieke T cellen voldoende gedeeld zijn veranderen ze en krijgen een door de APC geïnstrueerde gespecialiseerde functie, de T helper functie. Dit proces wordt ook wel differentiatie genoemd. Door deling en daarna differentiatie van een antigen-specifieke T cel ontstaat er dus een grote populatie gespecialiseerde T helper cellen die allemaal hetzelfde antigen herkennen. Deze T helper cellen zijn gespecialiseerd om hulp te bieden aan B cellen. B cellen zijn cellen die stoffen, antilichamen genaamd, kunnen maken welke belangrijk zijn bij de afweer. Net zoals de T cellen, bestaan er een grote hoeveelheid aan B cellen die allemaal voor een speciaal een antigen opgeleid zijn. Als B cellen die voor hetzelfde pathogeen (*pathogeen 3* in het voorbeeld **figuur 1**) opgeleid zijn als de T cel die hulp komt aanbieden dan verandert de B cel in een fabriek die antilichamen, ook wel immuunglobuline (IgG) genoemd, produceert. Antilichamen herkennen en binden aan de lichaamsvreemde stoffen of pathogenen zodat deze beter herkenbaar worden en een signaal geven aan de witte bloed cellen van het immuunsysteem dat zegt 'vernietig deze lichaamsvreemde stof'.



Figuur 1. Een T cel gemedieerde immuun response. *APC*: antigen presenterende cel; *Ag*: antigen; *TCR*: T cel receptor.

Nadat het pathogeen vernietigd is zijn veel van de gespecialiseerde cellen overbodig geworden en wordt de aanmaak ervan geremd zodat er slechts een paar T en B cellen overblijven die bij een volgende invasie van hetzelfde pathogeen sneller en efficiënter kunnen reageren (zgn. *memory* T- en B-cellen).

Costimulatie van T cellen

De herkenning van het antigen op de APC is het eerste signaal dat T cellen ontvangen om in actie te komen. Het eerste signaal wordt gegenereerd doordat het antigen wordt herkend door een daarvoor specifiek molecuul, de T cel receptor (TCR) genoemd. Voor het ingang zetten van de celdeling en differentiatie van de T cel zijn echter extra signalen nodig (**figuur 1**). Deze signalen (collectief 'signaal 2' genoemd) worden gegenereerd door een groep moleculen, de costimulatie receptoren genoemd, die zich bevinden op het oppervlak van de T cel. De APC die het antigen aanbiedt heeft namelijk een andere groep 'tegen'moleculen - liganden genaamd- op het oppervlak gebracht nadat het met de lichaamsvreemde substantie in aanraking is geweest. Binding van deze liganden aan de costimulatie receptoren genereert signalen die nodig zijn voor volledige activatie van T cellen dat leidt tot vermeerdering en verandering van de T cellen.

B7 costimulatoire moleculen

Een belangrijke familie van costimulatoire ligand-receptor paren zijn de B7-CD28- achtige moleculen. Op de APC komen B7-achtige liganden voor welke op de T cellen de costimulatie receptoren, de CD28-achtige receptoren, binden (zie **hoodstuk 2**, **figuur 1**). Afhankelijk van het type costimulatoire receptor genereert het een positief dan wel een negatief signaal. Positieve signalen zijn essentieel voor de afweer tegen lichaamsvreemde stoffen; negatieve signalen zijn belangrijk voor het bewaren van de balans zodat ongewenste immuunactivatie uitblijft (bijv. bij auto-immuun reacties). Of een T cel wel of niet wordt geactiveerd is afhankelijk van de optelsom tussen positieve en negatieve signalen. Eén van de belangrijkste costimulatoire receptoren is CD28. Deze receptor bevindt zich op de T cel en bindt aan zijn ligand B7.1 of B7.2 op de APC. In de aanwezigheid van een TCR signaal leidt binding van B7.1 en/of B7.2 aan CD28 binding tot een positief signaal in de T cel. Ter illustratie: Als T cellen geen CD28 hebben worden ze minder goed geactiveerd en gaan ze zelfs dood. Deze gezamenlijke signalen leiden tot activatie en deling van een antigen specifieke T cel, specialisatie in T helper cellen en zal uiteindelijk B cellen aan zetten tot IgG productie. Een ander zeer belangrijk costimulatoir molecuul is zes jaar geleden ontdekt en is ICOS (Inducible Costimulator) genaamd. ICOS lijkt veel op CD28 qua structuur en functie en heeft een vergelijkbare ligand op de APC, B7RP-1. Omdat ICOS en CD28 zoveel op elkaar lijken hebben we onderzocht of ICOS en CD28 vergelijkbare functies hebben in de verschillende fasen van een immuun respons: T celdeling, overleving van de T cel en T cel gemedieerde IgG productie.

Samenvatting van de onderzoeksresultaten

We hebben ontdekt dat ICOS net als CD28 betrokken is bij deling van de T cellen. In **hoofdstuk 3** staat een muis model beschreven dat een defect heeft waardoor alle T cellen ongeremd gaan delen. Zoveel, dat uiteindelijk deze muis daaraan overlijdt. Er was al bekend dat deze deling afhankelijk is van CD28. Als CD28 er niet is, dan vind deze overdadige celdeling niet plaats. In dit hoofdstuk laten we zien dat naast CD28, ICOS betrokken is bij deze overmatige deling. Als ICOS geblokkeerd werd vond er minder celding plaats. Ook was er minder vorming van gespecialiseerde T helper cellen, mogelijk als gevolg van deze verminderde hoeveelheid cellen. Dit betekent dat naast CD28, ICOS ook een belangrijke rol speelt in celdeling van muizen T cellen. Wel kwam uit deze studies naar voren dat CD28 beter in staat is T cellen te activeren dan ICOS. **Hoofdstuk 4** beschrijft dat deze conclusie ook geldt voor T cellen afkomstig van de mens. ICOS is niet alleen belangrijk voor de deling van humane T cellen, ook kan ICOS signalen in de T cel genereren die er mogelijk voor zorgen dat ze beter in leven blijven. In **hoofdstuk 5** is de rol van ICOS ten opzichte van CD28 in de immuun respons tegen een bacterie *Neisseria menigitidis* die nekkramp veroorzaakt getest. Er bleek dat voor de vorming van IgG na vaccinatie tegen deze bacterie zowel ICOS als CD28 van essentieel belang waren. Tevens kwam uit dit onderzoek naar voren dat voor de toepassing van mogelijke nieuwe en verbeterde stoffen voor vaccinaties de aanwezigheid van ICOS zelfs belangrijker is dan CD28. In het laatste hoofdstuk, **hoofdstuk 6**, tonen we in een diermodel aan dat CD28 cruciaal is voor het ontstaan van astma symptomen in de muis. In conclusie zijn er dus verschillen en overeenkomsten tussen CD28 en ICOS. Terwijl CD28 cruciaal is voor de het eerste signaal dat leidt tot T celdeling en overleving, is ICOS belangrijker direct na de initiatie, maar kan daar wel bijdragen aan de toename van de T cel populatie. Beiden moleculen zijn belangrijk bij IgG productie, hoewel de aanwezigheid van de liganden lijkt te bepalen welk molecuul wanneer het belangrijkst is. Hieruit komt naar voren dat het manipuleren van ICOS naast CD28 aantrekkelijk kan zijn in situaties waar beïnvloeding van het immuunsysteem gebruikt wordt om ziekten te behandelen; dan wel voor verhoging van de T cel functie voor optimale afweer tegen schadelijke stoffen of remmen van de werking van T cellen in geval van immuun overactivatie zoals bij auto-immune ziekten. Tevens kan in geval van personen die niet goed reageren op vaccinatie gekeken worden of het niet goed functioneren van ICOS hiervoor de reden kan zijn.

Dankwoord

Dankwoord

Ere wie ere toekomt: mijn dankwoord. Allereerst degene die dit promotie-onderzoek mogelijk heeft gemaakt: mijn co-promotor Mariëtte Oosterwegel (TCR, signaal 1, initiatie van T cel respons): creatief en met passie en zeer goed in de grote lijnen en het overzicht bewaren. Ik ben blij en trots dat we het samen hebben afgemaakt. Daar heeft co-promotor Kiki Tesselaar (CD27, T cel survival signaal) een belangrijke bijdrage aan geleverd. Kiki, met je pragmatische aanpak en verhelderende kijk op de dingen kon het project door blijven lopen. Geruststellende woorden van jou kant deden me altijd veel goed.

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Wat is een T cell zonder costimulatie? Elise (CD28, constitutief tot expressie, belangrijk voor de initiatie van activatie en effector functie van T cellen), aan jou heb ik veel te danken. Proeven doen samen met jou is een genot, is motiverend, inspirerend en vooral gezellig. Mijn chaos heeft in jouw mappen structuur gevonden. Nuchter 'dat doe je toch gewoon zo?' en oplettend 'wanneer moet het artikel eigenlijk terug naar J.I?'. Naast mijn paranimf en steun en toeverlaat in het lab heb ik veel gehad aan jouw steun buiten het lab. Annette, mijn andere paranimf (PI3K, intracellulair ICOS bindend molecuul, medieert downstream signal transduction pathways), jij bent niet makkelijk in maar een paar woorden te omschrijven: creatief, betrokken, liefdevol, gevoelig, veelzijdig en waardevol dekken de lading maar amper. Het is werkelijk een genot om jou als vriendin te hebben!

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Björn (B7RP-1, ligand voor ICOS), met jou wordt alles een beetje beter. Leuke dingen worden geweldig en minder leuke dingen worden draaglijker. Voor mij eerst oppervlakkige clichés hebben nu betekenis. Ik vind je heel bijzonder en ik vind het heerlijk om bij je te zijn nu en heel lang!

Miranda (ICOS)

Curriculum vitae

De auteur van dit proefschrift is geboren in Heesch op 18 februari 1975. Ze heeft het VWO doorlopen aan het Titus Brandsma Lyceum te Oss, waar ze in 1993 haar diploma behaalde. Hetzelfde jaar is ze haar studie Biologie aan de Katholieke Universiteit Nijmegen gestart. Na het propedeuse jaar heeft ze haar studie voortgezet in de differentiatie-richting Medische Biologie. Haar eerste hoofdstage betrof fundamenteel onderzoek aan TGFβ-gerelateerde groeifactoren bij Prof. Olijve op de afdeling Toegepaste Biologie op de Katholieke Universiteit Nijmegen. In haar tweede stage op de afdeling Molecular Medicine van Prof. Farzeneh, King's college University te Londen was ze betrokken bij de ontwikkeling van gentherapie om een efficiënte immuun respons tegen tumoren te genereren. Haar laatste stage was op de afdeling Oncology and Immunology in Newcastle (Australie). Onder directe begeleiding van Prof. Hersey heeft ze daar onderzoek gedaan naar de behandeling van melanoma door combinatie van cytostatica en TNF-gerelateerde receptoren. In november 1998 studeerde ze af, waarna ze het onderzoek nog heeft voortgezet als junior onderzoeker. In 1999 is ze teruggekeerd naar Nederland om in maart 2000 haar promotieonderzoek te starten op de afdeling Immunologie van het Universitair Medisch Centrum te Utrecht van Prof. J. van de Winkel en Prof. H. Clevers en later Prof. F. Miedema onder begeleiding van Dr. M. Oosterwegel en Dr. K. Tesselaar. Na het succesvol afronden van haar promotieonderzoek is zij per 1 maart 2006 aangesteld als postdoc bij de afdeling Hematologie (Prof. Ph.G. de Groot, UMC Utrecht) onder begeleiding van Dr. Martijn Gebbink. Hier verricht ze onderzoek naar eiwitmisvouwingsziekten.

List of publications

Van Berkel, E. A. T. and M. A. Oosterwegel. 2006. CD28 and ICOS: separate or similar costimulators of T cells? *Immunol.Lett.* in press.

Van Berkel, E. A. T., E. H. R. Schrijver, A. van Mourik, K. Tesselaar, P. van der Ley, L. Steeghs and M. A. Oosterwegel. 2005. ICOS-mediated costimulation predominates in the adjuvant function of *Neisseria meningitidis lpxL1* LPS. submitted.

Van Berkel, E. A. T., E. H. R. Schrijver, F. M. A. Hofhuis, A. H. Sharpe, A. J. Coyle, C. P. Broeren, K. Tesselaar and M. A. Oosterwegel. 2004. ICOS contributes to T cell expansion in CTLA-4 deficient mice. *J.Immunol.* 175(1):182-188.

Deurloo, D. T., van Berkel, M. A., B. C. van Esch, F. Hofhuis, F. P. Nijkamp, M. A. Oosterwegel and A. J. van Oosterhout. 2003. CD28/CTLA4 double deficient mice demonstrate crucial role for B7 co-stimulation in the induction of allergic lower airways disease. *Clin.Exp.Allergy* 33(9):1297-1304.

Franco, A. V., X. D. Zhang, E. van Berkel, J. E. Sanders, X. Y. Zhang, W. D. Thomas, T. Nguyen and P. Hersey. 2001. The role of NF-kappa B in TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of melanoma cells. *J.Immunol.* 166(9): 5337-45.

Gaken, J, J. Jiang, K. Daniel, E. van Berkel, C. Hughes, M. Kuiper, D. Darling, M. Tavassoli, J. Galea-Lauri, K. Ford, M. Kemeny, S. Russell and F. Farzaneh. 2000. Fusagene vectors: a novel strategy for the expression of multiple genes from a single cistron. *Gene Ther.* 7(23):1979-85.

Boersma, C. J. C., P. Kloen, M. Bloemen, J. M. A. Hendriks, E. A. T. van Berkel, W. Olijve and E.J.J. van Zoelen (1999) Homeobox proteins as signal transduction intermediates in regulation of NCAM expression by recombinant human bone morphogenetic protein 2 in osteoblast-like cells. *Mol.Cell Biol.Res.Commun.* 1:117-124.

List of abbreviations

AA	amino acid
Ab	antibody
Ag	antigen
alum	aluminium hydroxide
APC	antigen presenting cell
BALF	broncho-alveolar lavage
BCR	B cell receptor
BTLA	B and T lymphocyte attenuator
CTLA-4	cytotoxic T lymphocyte antigen-4
DC	dendritic cell
DIG	digoxigenin
DRC	dose-response curve
EU	experimental unit
GC	germinal center
HVEM	herpus virus entry mediator
ICOS	inducible costimulator
IFN-γ	Interferon gamma
IgG	immunoglobulin
IL-(2,4,10,17)	interleukin-(2,4,10,17)
LPS	lipopolysaccharide
OMC	outer membrane complexes
OVA	ovalbumin
PD-1	programmed cell death-1
P_{enh}	enhanced pause
TCR	T cell receptor
TLN	thoracic lymph node
TLR	toll like receptor
Treg	regulatory T cell
Th	helper T cell