

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CD28^{-/-} T cells can respond to ICOS-mediated costimulation, indicating that ICOS functions independently from CD28 (unpublished results) (2).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with the TCR, α CD28 was more effective in the induction of proliferation compared to α ICOS in both cell types (unpublished data).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 α ICOS 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-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 Ca²⁺ 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. meningitidis*, 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 maintenance 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). Maintenance 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 α ICOS 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

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