Contributions of CD27 and relatives to the specific immune response
Hendriks, J.A.

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Chapter 2

CD27/CD70 interactions are not required for T cell development or selection in the thymus, but promote deletion of self-specific T cells in the periphery.

Jenny Hendriks, Loes Gravestein, Yanling Xiao, and Jannie Borst

To be submitted
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Jenny Hendriks, Loes Gravestein, Yanling Xiao, and Jannie Borst

Division of Immunology, The Netherlands Cancer Institute; 1066 CX Amsterdam, The Netherlands

Summary

Expression of CD27 and CD70 in the thymus, as well as functional effects of anti-CD27 mAb in vivo previously suggested a role for this receptor/ligand pair in T cell development. However, thymic cellularity and thymocyte subpopulations were normal in CD27"~ mice. Also, pre-T cell expansion and differentiation to the CD4^8^+ stage, as induced by anti-CD3 mAb in RAG-1"~ mice was not affected by CD27-deficiency. Positive and negative selection of CD4^8^- thymocytes was also normal in CD27"~ mice, as tested in the H-Y TCR transgenic model. Effects of constitutive overexpression of CD70 on central and peripheral tolerance were assessed in transgenic mice with ubiquitous expression of the influenza virus nucleoprotein (NP). Constitutive stimulation of CD27 by transgenic CD70 did not break central tolerance to NP in this model, nor allow recruitment of NP-specific peripheral T cells that had escaped from clonal deletion into a responder T cell pool. Strikingly though, deliberate stimulation of CD27 promoted the disappearance of adoptively transferred NP-specific T cells from the circulation in NP transgenic mice. We conclude that CD27/CD70 are not important for thymic T cell development and selection, but can promote peripheral deletion of self-specific T cells.

Introduction

The Tumor Necrosis Factor (TNF) receptor family plays a key role in regulating life and death of lymphocytes. Its members are death receptors, as well as receptors that bind the so-called TNF receptor-associated factors (Trafs) (1). Traf-linked receptors such as CD27, CD30, CD40, OX40 (CD134), and 4-1BB (CD137), have been implicated in cell proliferation, differentiation, survival, and migration (2). Trafs signal to NF-κB and Jun kinase, but which genes the Traf pathways target, is mostly undefined (3). OX40 and 4-1BB have been directly linked to cell survival by the upregulation of anti-apoptotic proteins Bcl-2, Bcl-xL and Bfl-1 (4,5). Also CD27 has now been found to promote the expression of
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Bcl-xL, at least in human T cells (M. van Oosterwijk and R van Lier, personal communication).

Our studies focus on CD27, which is present on most naive CD4+ and CD8+ T cells (6,7). CD70, the ligand of CD27, is exclusively expressed on activated lymphocytes and mature dendritic cells (DC). CD70 is induced by TCR triggering on T cells and its expression is modulated by cytokines (8,9). On DC, CD40 interactions with its ligand or “danger” signals such as LPS can induce CD70 expression (9,10). CD27 has been shown to provide a co-stimulatory signal to T cells that promotes TCR-induced T cell expansion in vitro (7,11).

Upon intra-nasal infection with influenza virus, CD27−/− mice showed impaired generation of antigen-specific T cells and accumulation of CD4+ and CD8+ T cells at the effector site, i.e. the lung. The impact of CD27 deletion was most dramatic in the memory response (12). In CD70 transgenic mice the reverse phenotype can be found. In these mice, T cells with an effector phenotype accumulate (13,14). Comparing the relative contributions of CD27 and the well-studied co-stimulatory molecule CD28, we found that primary and memory CD8+ T cell responses to influenza virus are dependent on the collective contribution of both receptors. Without affecting cell cycle activity, CD27 supported the survival of activated T cells throughout successive rounds of division at the site of priming and the site of infection (15). CD27, like OX40 and 4-1BB, is essential to the generation of memory CD8+ T cells and determines the magnitude of CD8+ effector T cell accumulation in the secondary response (Hendriks et al, submitted).

In addition to the role of CD27 in supporting antigen-specific T cell responses, CD27 may impact on T cell development. CD27 expression is induced by pre-TCR signalling and persists throughout thymic development. CD70 has also been found on cells in the thymic medulla, which may be epithelial in origin (9,16). In vivo infusion of anti-CD27 antibody abrogated anti-CD3-driven pre-T cell expansion and differentiation in RAG-1−/− mice, inspiring the conclusion that CD27 contributes to pre-TCR mediated thymocyte development (17).

Other members of the TNF receptor family have been implicated in T cell development. CD40-CD154 interactions are indispensable for negative selection. In the absence of CD40 signals, either by antibody-mediated blocking or in CD40L deficient mice, negative selection of CD4+ T cells was defective (18-20). Other studies showed, that expression of CD40 on thymic B cells was crucial to support negative selection (21,22). The role of CD30 in thymocyte selection is subject to debate. In a TCR transgenic model, CD30−/− thymocytes showed impaired negative selection (23). Accordingly, transgenic CD30 overexpression revealed enhanced negative selection induced by bacterial superantigen and specific peptide, which could be counteracted by transgenic Bcl-2 expression (24). In contrast, others found no evidence for essential requirement for CD30 in class-I and -II restricted negative selection (25).
CD27 promotes deletion of self-specific T cells

Targeted deletion of receptors in mice is an optimal approach to determine the exact contribution each TNF receptor family member makes in controlling the immune system. To elucidate the potential role of CD27 in T cell development, we studied a CD27"/" mouse strain. Here, we present effects of CD27-deficiency on thymocyte selection and central and peripheral tolerance. Our analysis reveals that CD27/CD70 interactions are not required for T cell development or positive and negative selection of the thymic repertoire. However, while central tolerance could not be altered by enforced CD27/CD70 interactions, deletion of self-specific T cells in the periphery appeared to be promoted.

Results

T cell development in CD27-deficient mice is normal.

No differences in cellularity of the thymus between wild type and CD27"/" mice were detected. The size of CD4^8^, CD4^8^- and mature CD4^8^- and CD4^8+^ populations (Fig. 1), as well as subset composition of the CD4^8^- compartment (not shown) are as in wild-type mice.

We previously found that injection of anti-CD27 mAb in vivo inhibits generation of the CD4^8+^ thymic compartment (17). This suggested that CD27 provides a co-stimulatory signal for pre-TCR-induced thymocyte development. However, we now find that CD27 deletion does not affect cellularity and composition of the thymus. Possibly, the CD4^8+^ compartment in CD27"/" mice is generated by a selected cell population, which is able to bypass CD27 requirement. To test this, the disrupted CD27 allele was crossed onto the RAG-1"/" background, in which T cell development is

![Figure 1. Thymocyte populations are normal in CD27 deficient mice.](image)

(Left panel) Single cell preparations of 4 thymi of 8 week old CD27"+/" and CD27"/-" mice were counted. Mean numbers (#) of thymocytes per thymus are indicated. Error bars denote standard deviations. (Right panel) Thymocytes were double stained with mAbs to CD4 and CD8 and fluorescence intensity (FI) was determined by flow cytometry (middle and right panel). Shown are representative dotplots of at least four independent analyses.
arrested at the CD44^25^+ CD4^8^- stage due to lack of pre-TCR expression (26). In these mice, no selection has taken place for cells able to make the transition to the CD4^8^+ stage. CD27^+/+^ and CD27^-/-^ RAG-1-deficient mice were treated with anti-CD3e mAb, which mimics pre-TCR signalling (27), in the presence or absence of anti-CD27 mAb. After 4 days, thymi were analysed by flow cytometry. As shown in Fig. 2, anti-CD3e mAb induced generation of the CD4^8^+ compartment equally well in wild-type and CD27^-/-^ RAG-1-deficient mice. This experiment indicates that CD27 is not required for pre-T cell expansion and differentiation. It also underlines the specificity of the effect of anti-CD27 mAb, since this antibody inhibited CD3e-induced generation of the CD4^8^+ compartment in CD27^+/+^, but not in CD27^-/-^ mice (Fig. 2). We conclude that anti-CD27 mAb specifically inhibits pre-TCR induced thymocyte development, but the mechanism by which this inhibition occurs differs from that proposed previously: The antibody does not block a CD27/CD70 interaction that is a prerequisite for pre-TCR signalling to be effective.

**CD27 deletion does not affect positive and negative thymocyte selection.**

Since TNF receptor family members are generally implicated in regulation of cell survival, we examined whether CD27 is involved in thymocyte repertoire selection. In the mouse, CD27 is expressed on all CD4^8^+ thymocytes and CD70 has been detected on cells in the thymic medulla, which may be epithelial in origin (9,17). Therefore, CD27/CD70 expression seems consistent with a function in thymocyte selection. To examine this, the mutated CD27 allele was crossed onto a background transgenic for a TCRαβ with specificity for the male antigen H-Y, restricted by H2-D^b^ (28). To introduce transgenic T cell precursors in a selecting MHC background, bone marrow from TCR transgenic CD27^-/-^
Figure 3. Thymic repertoire selection in CD27+/+ and CD27−/− H-Y TCR transgenic mice. Bone marrow was harvested from H-Y TCR transgenic CD27+/+ and CD27−/− littermates and injected into irradiated male and female RAG-1−/− mice of the H2b haplotype. After 8 weeks, thymocytes were counted and stained with T3.70-FITC, anti-CD4-PE and anti-CD8-Tricolor. T3.70+ cells were gated and CD8+, CD4+, CD8/4+, or CD8/4− populations were quantitated within the TCR transgenic pool. (A) Representative flow cytometric analysis of CD4 and CD8 expression within the TCR transgenic thymocyte population. Total number of cells per thymus is indicated to demonstrate effectiveness of thymic reconstitution and negative selection. (B) Distribution of TCR transgenic thymocytes over thymic subpopulations. Each bar indicates means and standard deviations of the number of cells of the CD4−8−, CD4+8−, CD4+8+ or CD4−8+ phenotype per thymus, based on analysis of 4 recipient mice.

and CD27+/+ mice was transferred to lethally irradiated male and female RAG-1−/− mice of the H2b haplotype. After 8 weeks, population of the thymus was examined by cell counting and phenotypic analysis. In female mice, effective generation of all thymocyte populations had taken place by this time. The TCR transgenic CD8+ mature subset was increased in cellularity as compared to the non-transgenic situation, consistent with positive selection of CD4+8− thymocytes capable of recognising the H-Y/H2-Db complex (Fig. 3). In male mice, thymic cellularity was dramatically reduced, with depletion of the CD4+8+ and mature CD8+ and CD4+ compartments, consistent with negative selection of transgenic thymocytes at the transition from the CD4−8− to the CD4+8+ stage (Fig. 3). There was no difference in the composition of thymocyte subsets in wild-type versus CD27−/− mice, be it males or females. The same results were obtained when thymic selection was determined in mice crossed to express the transgenic TCR, homozygous CD27 wild type or mutant alleles, and the appropriate H2-Db restriction element (data not shown). We conclude therefore, that CD27 does not affect the outcome of positive or negative thymocyte selection in this TCR transgenic system.
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Constitutive CD70 expression on B cells does not break central tolerance.
To study possible effects of deliberate CD27 ligation on thymic selection, we made use of the availability of CD70 transgenic (tg) mice. CD70tg mice, which constitutively express CD70 on B cells, show deregulated T cell homeostasis, which is dependent on TCR triggering (13,14). We crossed CD70tg mice with transgenic mice that ubiquitously express a fragment (amino acids 1,2, and 328-498) of the influenza nucleoprotein (NP) under control of the H-2K promoter. These mice have been shown to delete NP-reactive CD8+ T cells in the thymus and display tolerance for NP (28). Low avidity NP-specific CD8+ T cells that escape negative selection in this model cannot expand in vivo upon exposure to influenza virus A/HK/1/68, which expresses the immunodominant NP366-374 ASNENMDAM peptide present in the transgene, but can respond to altered peptide ligands (29,30).

Wild-type mice, mice expressing CD70, or NP transgenes only, and mice expressing both CD70 and NP transgenes were intranasally infected with influenza A virus strain A/HK/1/68. At day 9, when the T cell response in the respiratory tract peaks (12), lungs were harvested and cell suspensions derived from them were analysed for the presence of CD8+ T cells specific for the NP366-374 peptide, using MHC class I tetramers. As also demonstrated previously (31), higher numbers of virus-specific T cells were found at day 9 after infection in CD70tg mice than in wild-type mice (Fig. 4A). This indicates that deliberate CD27 triggering by CD70, as constitutively expressed on B cells, enhanced the expansion and/or maintenance of virus-specific CD8+ T cells. In NPtg mice, virus-specific T cells were extremely low (Fig. 4A). Staining was in fact comparable to background staining by tetramers in naïve mice (data not shown). Deliberate CD27 triggering could not break tolerance in NPtg mice, since the number of virus-specific CD8+ T cells found in the lung at the peak of the infection was equally low in presence or absence of transgenic CD70 expression (Fig. 4A).

To further examine the extent of tolerance, we assessed the presence and responder capacity of NP366-374-specific CD8+ T cells by in vitro re-stimulation with the immunodominant ASNENMDAM peptide. At day 9 after influenza virus infection, spleen cells were harvested derived from the mice analysed in Fig. 4A, and re-stimulated in vitro for 2 weeks with NP366-374 peptide. Thereafter, responder T cells were tested for IFN-γ production by intracellular immunofluorescence. The proportion of wild-type and CD70tg T cells in the culture that could produce IFN-γ was directly correlated with the dose of NP peptide used for stimulation. In line with the enhanced in vivo response, percentages of IFN-γ positive cells were higher in the CD70tg culture than in the wild-type culture (Fig.4B). However, no IFN-γ producing cells could be detected in the cultures derived from NPtg mice, regardless of the constitutive expression of CD70. We conclude that deliberate CD27 stimulation by CD70 on B cells cannot break central tolerance. It can also not recruit
CD70 promotes deletion of self-specific T cells

**Figure 4. Constitutive CD70 expression does not affect tolerance in the NP transgenic model.**

Wild type (WT), NPtg, CD70tg and NPtg/CD70tg mice were infected with 0.2 HAU A/HK/1/68 influenza A virus. Nine days later, mice were sacrificed and lungs were harvested for analysis. Single cell preparations of lungs were stained with anti-CD8 mAb and H2-D^b/NP_{366-374} tetramers and analysed by flow cytometry. (A) Absolute numbers of virus-specific CD8^+ T cells were calculated from the percentage of tetramer^+CD8^+ cells and the total number of lymphocytes in the preparation. Means and standard deviations are shown from 3 mice per group. The experiment is representative of two. (B) Spleen cells from mice in (A) were harvested at day 9 after influenza virus infection, pooled and re-stimulated in vitro. After two weeks, cells were stimulated with indicated concentrations of peptide and intracellular IFN-γ was measured by immunofluorescence. The experiment is representative of two.

CD8^+ T cells that escape from central deletion in NP-transgenic mice into an antigen-specific effector T cell pool.

**Peripheral deletion of self-reactive T cells is altered by CD27/CD70 interactions**

To examine the role of CD27/CD70 interactions in the deletion of self-reactive T cells in the periphery, we crossed CD27^-/- mice to F5 TCR transgenic mice (32). These mice carry a TCR that is specific for the NP_{366-374} peptide and it has been shown that these T cells are negatively selected in the thymus of NPtg mice (29,32). Injection of mature F5 T cells into NPtg mice was previously shown to result in initial activation and expansion of these T cells in the spleen, followed by their disappearance (33). To test whether the presence of CD27 on self-specific T cells affected their response to self antigen, we compared proliferation and accumulation of purified F5/CD27^+/+ or F5/CD27^-/- T cells upon their injection into wild-type or NPtg mice. To follow proliferation, T cells were labelled with carboxy-fluorescein diacetate succinimidyl ester (CFSE) prior to adoptive transfer. In the first three days following transfer into wild-type recipients, numbers of F5 T cells present in the circulation (blood) did not change and cells did not divide, since CFSE remained undiluted (Fig. 5A). In NPtg mice, however, F5 T cells were virtually undetectable in the blood at days 1 and 2 after adoptive transfer, while at day 3 significant numbers appeared,
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A

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Figure 5. Transgenic expression of CD70 during peripheral deletion of self-reactive T cells.

Purified splenic T cells that were CFSE labelled from F5 or F5/CD27\(^+\) mice were injected into wildtype (WT) or NPtg mice with or without CD70tg B cells. (A) On days indicated blood was taken from the tail vein and stained with anti-CD8 mAb and H2-D\(^b\)/NP\(_{366-374}\) tetramers. Shown are representative CFSE/tetramer dotplots of CD8\(^+\) T cells. (B) Four weeks after the adoptive transfer described in (A), recipient mice were infected with 0.2 HAU A/HK/1/68 Influenza A virus. Nine days after infection, lungs were harvested and stained with anti-CD8 mAb and H2-D\(^b\)/NP\(_{366-374}\) tetramers and analysed by flow cytometry. Dotplots are of pooled lung samples of three mice per group. (C) From percentages in (B) and numbers of total lymphocytes in the lung, absolute numbers of H2-D\(^b\)/NP\(_{366-374}\) tetramers binding T cells were calculated. Results are of pooled lung samples of three mice per group. Experiment is representative of two.
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which all had extensively proliferated according to CFSE dilution (Fig. 5A). At day 7 after transfer, these responder cells had fully disappeared from the circulation in NPtg mice, while in wild-type recipients F5 T cells could still be detected at this time point (Fig. 5A). The behaviour of CD27-deficient F5 T cells was very similar to that of wild-type F5 T cells in both wild-type and NPtg recipients (Fig. 5A). Moreover, deliberate triggering of CD27 by co-injection of CD70tg spleen cells did not detectably alter the behaviour of F5 T cells in wild-type or NPtg recipients (Fig. 5A).

To test whether F5 T cells were still present in NPtg mice and could be activated by viral infection, we infected the same mice depicted in Fig. 5A intranasally with influenza virus A/HK/1/68 four weeks after adoptive transfer and read out the T cell response in the lung at day 9 after infection. Clearly, a significant H-2D$^\text{b}$/NP$_{366-374}$-specific CD8$^+$ T cell response took place in wild-type mice that had received either wild-type or CD27$^{-/-}$ F5 T cells, according to tetramer staining of lung-infiltrating cells (Fig. 5B and 5C). We could not distinguish wild-type recipient cells from F5 T cells in this assay. However, endogenous responses, expressed in absolute numbers of H2-D$^b$/NP$_{366-374}$ tetramer binding T cells, are at least 5 times lower than the response observed here (12,15). This indicates that adoptively transferred F5 T cells contributed the major part of the response. In NPtg mice that had received F5 T cells four weeks earlier a response to influenza virus could be detected, although it was greatly reduced as compared to the response in wild-type recipients (Fig. 5B,C). The response observed must be attributed to adoptively transferred F5 T cells, since endogenous H2-D$^b$/NP$_{366-374}$-specific T cells do not respond to A/HK/1/68 virus in NPtg mice (Fig. 4A). CD27-deficiency apparently enhanced the potential of NP$_{366-374}$-specific T cells to respond to infection in NPtg recipients (Fig. 5B,C). This suggests that CD27 on self-specific T cells promotes their deletion. Interestingly, the addition of CD70tg spleen cells in the transfer led to a complete absence of wild-type F5 T cell responses in NPtg mice (Fig. 5B,C). Apparently, deliberate CD27 stimulation promoted the deletion of self-specific T cells. Surprisingly, also CD27-deficient F5 T cells failed to respond to virus infection when CD70tg B cells were present in recipient mice. We conclude that CD27/CD70 interactions can promote the elimination of self-specific T cells from the responder T cell pool in the periphery. This is apparently not only accomplished by triggering CD27 on T cells, but also by interaction of CD70 with CD27 on non-T cells.

Discussion

We find that CD27 is not essential for generation and maintenance of thymocyte populations. Earlier, we described that the size of mature T and B cell populations in peripheral organs of non-immunized mice were unaffected by CD27 deletion (12). We had previously hypothesized that CD27 acts as a co-stimulus for pre-TCR function (17), based on the inhibitory effect of anti-CD27 mAb on generation of the CD4$^+$8$^+$ thymic
compartment. However, our follow-up experiments as described in this paper established that CD3-driven T cell development is normal in CD27-deficient mice. We conclude therefore that CD27/CD70 interaction is not required to support pre-TCR function. Contrary to our previous hypothesis, in vivo administered anti-CD27 mAb apparently does not inhibit endogenous CD27/CD70 interactions required for pre-T cell expansion and differentiation. Given our present findings, we must conclude that this antibody triggers a non-physiological CD27 function that counteracts the endogenous pre-TCR-induced response.

To test whether CD27 regulates thymocyte apoptosis, we studied repertoire selection in TCR transgenic mice specific for the male antigen H-Y (28). Absence of CD27 did not alter selection, as assayed at steady state. It should be remarked that kinetic experiments, that might reveal modest effects of CD27, were not performed. Also, choice of thymocyte selection model and the transgenic TCR may influence the result. Whether TNF receptor family members are generally important for T cell repertoire selection in the thymus is unclear. Injection of anti-CD3 mAbs and in vitro culture with isolated antigen presenting cells indicated the involvement of TNF receptors in negative selection. However, in TNF receptor deficient mice, no defect in negative selection was found using H-Y or AND TCR transgenic mice (34). CD40 and CD30 have been implicated in thymocyte selection using gene targeted mice (18,23). CD40 may regulate levels of CD28 expression in the thymus (18), which is important for negative selection according to certain studies (35,36), but not according to others (37,38). The role of CD30 in thymocyte selection also remains debated (25).

Negative selection of potentially self-specific T cells leads to centrally defined tolerance. We found that constitutive CD70 expression on B cells could not break central tolerance and alter the repertoire that is selected in the thymus. Providing excess CD70 on B cells in the system may not be optimal to influence thymic selection, since normally, CD70 non-B cells in the thymus (9). However, studies on CD40 show that expression of CD40 is required on thymic B cells for adequate negative selection (21,22). Precise analysis of CD70 expression in the thymus should yield results on which cells express CD70 and indicate on when and where CD27/CD70 interactions may come into play in thymic processes. Alternatively, transgenic expression of CD70 on DC may reveal a role for CD27 in thymic selection.

The process of negative selection is not absolute and self-specific T cells may avoid deletion and reach the periphery (39). These self-specific T cells do not cause autoimmunity since several mechanisms keep them in check, leading to peripheral tolerance. T cells may be unresponsive upon antigen encounter (anergic). They may downregulate their TCR and subsequently be ignorant for the antigen. Alternatively, self-specific T cells may be deleted by apoptosis in the periphery. We found that constitutive
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CD70 expression on B cells did not affect the non-responsiveness of NP-specific CD8+ T cell repertoire that is still present in NPtg mice. Upon infection with influenza virus carrying the NP366-374 epitope that is present in the transgene, these T cells could still not be activated. When NP-specific F5 TCR transgenic T cells were introduced into the bloodstream NPtg mice, these cells were found to initially disappear from circulation and to reappear after extensive division two days later. After 7 days, the transferred self-specific had disappeared from the peripheral blood stream by an unidentified mechanism. Unexpectedly, absence of CD27 on the F5 T cells enhanced their responsiveness after influenza virus infection. It remains to be established whether these cells relocated to tissues and/or were eliminated by apoptotic cell death.

We have previously shown in the influenza virus model that CD27 promotes survival of activated CD8+ T cells in draining lymph nodes and thereby enhances the accumulation of virus-specific T cells at the site of infection (15). Therefore, it is unlikely that in NPtg mice lack of CD27 on F5 T cells favoured their expansion upon influenza infection. Probably, CD27-deficient F5 T cells were less effectively deleted from the circulation than wild-type F5 T cells prior to influenza virus infection. This is in line with the observation that F5 T cells were more effectively deleted in presence of constitutive CD70 expression on transgenic B cells. It remains to be established by which mechanism adoptively transferred NP specific T cells are eliminated from the circulation in NPtg mice and how CD27/CD70 interaction promotes this. Transgenic CD70 did not only act by triggering CD27 on F5 T cells to cause deletion, since CD27−/− F5 T cells also disappeared. Therefore, CD70 expression must have conditioned other cells to bring about the disappearance of F5 T cells.

In certain forms of peripheral tolerance, T cells are not deleted but rendered unresponsive (40). CD25+ regulatory T cells are essential for maintaining this type of tolerance, since depletion of this subset leads to autoimmune disease (41). These suppress autoreactive T cell responses via cytotoxic T lymphocyte associated antigen 4 (CTLA-4), a negative regulatory CD28 homologue (42,43). However, in NPtg mice, deletion of self-reactive T cells by apoptosis is more likely to play a role in peripheral tolerance than suppression, since adoptively transferred F5 cells became undetectable. Peripheral deletion of self-specific T cells by apoptosis critically depends on CD95 (44). Studies in the CD70 transgenic mouse show that CD70 triggering causes upregulation of the CD95 receptor and that these cells are more susceptible to CD95-mediated apoptosis (Arens et al., to be submitted). Crossing the CD70 transgene into CD95-deficient lpr/lpr mice, exacerbates the lympho-proliferative disease that these mice have and leads to early death by 3-4 weeks of age. To uncover CD95 as the mediator by which CD27 promotes deletion of self-specific T cells, F5 TCR transgene could be crossed into the lpr/lpr mice.
We have not found a role for CD27 in T cell development or thymocyte selection. However, CD27 may regulate peripheral tolerance by promoting deletion of chronically activated T cells, such as self-specific T cells. We propose that CD27 signals, in addition to supporting the survival of antigen-activated T cells, sensitize these cells for CD95-mediated apoptosis, leading to a regulated involution of the expanded T cell population. With this negative feedback loop, Traf-binding and death receptor members of the TNF receptor family regulate the balance of expansion of antigen-specific T cells and prevention autoimmunity by self-specific T cells.

**Experimental Procedures**

**Mice**

All mice were bred in the facility of the Netherlands Cancer Institute under specific pathogen-free conditions and used for experiments at 6-10 weeks of age. Generation and analysis of CD27 mice was described earlier (11). RAG-1 mice (26) back crossed to the H2b background were provided by Dr. J. Kirberg. H-Y TCR transgenic mice (28) were provided by Drs. J. Kirberg and H. von Boehmer (Basel Institute for Immunology, Basel, Switzerland). NP- (29) and F5 TCR transgenic (32) mice were provided by Dr. D. Kioussis (National Institute for Medical Research, The Ridgeway, Mill Hill, London). CD70 transgenic mice were provided by Dr. R.A.W. van Lier (Academic Medical Center, Amsterdam, The Netherlands) (13).

**Genotypic analysis of mice**

The mutant RAG-1 allele was detected by a PCR on the neomycin resistance gene using nucleotides 394-413 as sense- and 1048-1066 as anti-sense primer (26). The wild type RAG-1 allele was detected using nucleotides 531-552 as sense- and 1276-1296 as anti-sense primer (45). H-Y transgenic mice were analysed for TCR transgene expression by staining peripheral blood T cells with FITC-conjugated anti-TCR mAb T3.70 (28). Similarly, F5 transgenic T cells were detected with anti-V*p1 mAb. NPtg mice were analysed by performing PCR on tail DNA (29). CD70tg B cells were detected with anti-CD70 mAb provided by R.A.W. van Lier. CD27 mice were analysed using anti-CD27 mAb LG.3A10 to stain peripheral blood cells (11).

**Flow cytometry**

Peripheral blood was collected and cells were spun down, followed by lysis of erythrocytes on ice for 10 min in 0.14 M NH₄Cl, 0.017 M Tris-HCl, pH 7.2. Peripheral blood cells and cells from bone marrow, thymus, spleen and lymph nodes were pre-incubated with Fe Block (anti-CD16/CD32 mAb 2.4G2, Pharmingen) and washed in staining buffer (PBS, 0.5% BSA, 0.01% sodium azide). Next, they were incubated with specific antibody directly conjugated to FITC, phycoerythrin (PE) or biotin as indicated, washed, incubated with streptavidin-Tricolor or streptavidin-PE (Caltag, San Diego, CA) when applicable, washed and analysed using a FACScan and LYSYS II software (Becton Dickinson, Mountain View, CA). mAb used for immunofluorescence were obtained from Pharmingen or donated by investigators. mAbs were anti-CD3e, 500A2; anti-CD4, RM4-5; anti-CD8a, 53-6.7; anti-CD27, LG.3A10; anti-CD28, 37.51; anti-CD44, IM7; anti-CD45R/B220, RA3.6B2; anti-CD69, H1.2F3; anti-IgM, R6-60.2; anti-H-Y TCR, T3.70; anti-IFN-γ, XMG1.2. Allophycocyanin (APC)-labelled tetramers, comprising the murine class I molecule H2-Db, β2 microglobulin and the influenza
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Nucleoprotein (NP) peptide ASNENMDAM (NP366-374) were prepared as described (46,47). Cells were incubated with MHC tetramers together with anti-CD8α-FITC. Cells were washed in FACS buffer and analysed using a FACSCalibur and CELLQuest software (Becton Dickinson, Mountain View, CA).

Antibody treatment in vivo
CD27<sup>−/−</sup> mice descending from chimera x FVB crosses were bred with RAG-1<sup>−/−</sup> mice. Mice that were RAG-1<sup>−/−</sup> and either wild type, heterozygous or homozygous for the CD27 mutant allele were injected intravenously with 50 µg of anti-CD3ε mAb 145-2C11 with or without 50 µg of anti-CD27 mAb LG.3A10 on day 0. On day 4, mice were sacrificed and the absolute number and CD4/CD8 profile of thymocytes was determined.

Preparation of bone marrow chimeras
Bone marrow was harvested from femurs of CD27<sup>+/+</sup> and CD27<sup>−/−</sup> littermates, transgenic for the H-Y TCR. Cells were incubated with 2 µg per 10<sup>6</sup> cells of anti-Thy1.1 mAb for 30 min on ice, followed by extraction with 8 x 10<sup>6</sup> goat anti-rat Ig magnetic beads (Advanced Magnetics Inc., Cambridge, MA) per 10<sup>6</sup> cells. These T cell depleted bone marrow cells were injected intravenously at 3 x 10<sup>6</sup> cells per mouse into lethally irradiated (9 Gy) male and female RAG-1<sup>−/−</sup> mice of the H2<sup>b</sup> haplotype. Cells from one donor mouse were injected into two male and two female recipient mice. After 8 weeks, recipient mice were sacrificed and thymocytes were counted and phenotyped.

Virus infection
Influenza virus strain A/HK/1/68 was obtained from the Department of Virology, Erasmus University Rotterdam, The Netherlands. Mice were anaesthetized and infected intra-nasally with 50 µl Hank’s balanced salt solution containing 0.2 haemagglutinin units.

Restimulation in vitro and IFN-γ detection
Spleen cells from mice were pooled and re-stimulated in vitro with 0.0005 µg/ml influenza nucleoprotein (NP) peptide ASNENMDAM (NP<sub>366-374</sub>). After two weeks, cells were stimulated with concentrations of peptide ranging from 0 to 1.10<sup>-2</sup> µg/ml as indicated in the presence of 10 µg/ml Brefeldin A for 4 hours. Intracellular IFN-γ was detected by staining cells with anti-IFN-γ mAb in staining buffer containing 0.1% saponin and analysed by flow cytometry.

Adoptive transfer
T cells were purified from spleens of F5/CD27<sup>+/+</sup> and F5/CD27<sup>−/−</sup> mice by dissociating them in Iscove’s Modified Dulbecco’s medium (IMDM) supplemented with 8% FCS, penicillin and streptomycin. Erythrocytes were lysed in 0.14 M NH₄Cl, 0.017 M Tris-HCl, pH 7.2 for 5 min on ice. Cells were washed in medium, passed over nylon wool (Polysciences, Warrington, MA) and incubated on ice for 30 min with anti-MHC class II mAb M5/114. This was followed by a 30 min incubation on ice with 100 µl and 20 µl per 10<sup>7</sup> cells of goat anti-mouse Ig- and goat anti-rat Ig-coated magnetic beads (Advanced Magnetics Inc.), respectively. Purity of the resulting cell population was checked by immunofluorescence analysis with anti-CD3ε and anti-B220 mAbs. Only preparations that contained more than 98% T cells were used. T cells used for adoptive transfer were labeled with CFSE (Molecular Probes Inc. Eugene, OR). Cells were incubated at a concentration of 5 x 10<sup>7</sup> cells/ml in PBS, containing 0.1% BSA and 5 µM CFSE,
for 10 min at 37°C. Labeling was quenched with 10 ml of cold medium with 10% FCS and cells were washed twice with IMDM with 8% FCS prior to use. \(10 \times 10^6\) Purified, CFSE labeled F5/CD27+/+ or F5/CD27−/− T cells were suspended in 200 μl Hank’s balanced salt solution and injected intravenously into wild-type or NPtg mice with or without \(40 \times 10^6\) CD70tg spleen cells.

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**References**

CD27 promotes deletion of self-specific T cells


