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Chapter 2

HVEM does not compensate for CD27 deficiency during αβ T cell development and function

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ABSTRACT

In developing murine thymocytes, CD27 expression is upregulated by pre-TCR signaling. Moreover, antibody intervention studies suggested a role for CD27 at the pre-TCR checkpoint. Subsequent analysis of CD27-/- mice, however, revealed no role for CD27 in the thymic development of αβ T cells. We reconsidered the role of CD27 in T cell development when gene array revealed an upregulation of its close relative HVEM in naïve CD27-/- T cells. Membrane expression of HVEM was increased in CD27-/- thymocytes at the pre-TCR checkpoint, as well as in mature CD4+ and CD8+ thymocytes and peripheral T cells. We tested the possibility that HVEM compensated for CD27-deficiency during αβ T cell development by analyzing wild-type, CD27-/-, HVEM-/- and CD27-/-HVEM-/- mice. In all mouse strains, thymocyte populations were normal in size, excluding a redundancy between CD27 and HVEM. The impact of increased HVEM expression on CD27-/- T cell function was investigated by comparing the response of CD27-/- and CD27-/-HVEM-/- T cells to influenza virus infection. Additional HVEM deficiency did not alter the response of CD27-/- CD8+ T cells in terms of accumulation and cytokine expression. We conclude that HVEM does not compensate for the lack of CD27 during αβ T cell development, nor does it contribute to the impaired CD8+ T cell responses that were reported for CD27-/- mice.

INTRODUCTION

TNF receptor family members are well known for their effects on immune cell function. A number of these receptors act as T cell costimulatory molecules that promote the generation of effector and memory T cell populations (1). One of the prominent features of TNF receptor family members is their capacity to activate the NF-κB pathway that is potently anti-apoptotic (2). A main mechanism by which these receptors affect T cell function is therefore to promote their survival (1). Possibly depending on the cellular niche in which this occurs, TNF receptor family signaling may also have effects on cellular differentiation programs (3). In this respect, it is interesting that the NF-κB pathway can also regulate the expression of chemokines, which may allow cells to create niches in which their survival and differentiation are directed (4). While Lymphotoxin and TRANCE in this way have a profound impact on the formation of secondary lymphoid organs (5), impact of TNF receptor family members on thymic architecture, thymocyte development or selection seems limited. Thus far, only a role for CD40 in promoting negative selection of CD4+ thymocytes was revealed (6-8), while observations that CD30
may have a similar effect are debated (9-11). TNF receptor family member CD27 is known as a T cell costimulatory molecule that controls the survival of effector T cells (12-14). CD27 is expressed on the great majority of naïve CD4+ and CD8+ T cells in both human and mouse and comes into play when T cells recognize antigen on professional antigen presenting cells (APC), such as dendritic cells (15). CD27 function is controlled by the transient availability of its ligand CD70 that is under control of antigen receptor-, Toll-like receptor- and CD40 signaling and therefore is generally expressed on professional APC and T cells upon infection (16). The expression pattern of CD27 and CD70 in the thymus has suggested a role for CD27 in T cell development. In both human and mouse, CD70 is constitutively expressed on medullary thymic epithelial cells (mTEC), where selection of the T cell repertoire takes place (16,17). In human, CD27 is induced at the CD4+8+ (double positive, DP) stage of thymocyte development and hallmarks functional mature T cells (18). In the mouse, however, CD27 is already expressed at the early, CD4-8- (double negative, DN) stage of T cell development. Taghon et al. (19) showed that in the mouse, CD27 expression is high at the DN1 stage of thymocyte development and hallmarks functional mature T cells (18). In the mouse, however, CD27 is already expressed at the early, CD4-8- (double negative, DN) stage of T cell development.

Taghon et al. (19) showed that in the mouse, CD27 expression is high at the DN1 stage of thymocyte development, but rapidly decreases throughout the DN2 and DN3 stages. In the DN3 stage, two populations can be distinguished, one with low CD27 expression (DN3a) and one with high CD27 expression (DN3b). The authors showed that DN3b thymocytes are much more efficient in generating CD4+8+ double positive (DP) progeny and suggested that CD27 is essential for progression of DN3 cells to the DP stage (19). This is the checkpoint that is governed by the pre-TCR, which gives signals for thymocyte proliferation and differentiation and drives the development of the DP thymocyte compartment (20).

The findings by Taghon et al. tied in with an earlier observation by our group that in the mouse thymus, CD27 expression was upregulated by pre-TCR signaling and subsequently maintained throughout T cell development (21). To test a possible role of CD27 at the pre-TCR checkpoint, we mimicked pre-TCR signaling by infusion of anti-CD3 antibody in recombination-deficient RAG-1-/- mice (22). This drives the development of the DP thymocyte compartment from DN precursors in absence of functional (pre-)TCR expression (23). Co-injection of anti-CD27 antibody inhibited this process. Moreover, it also depleted the DP compartment in TCR-/- mice. From these data, we suggested that in the mouse, CD27 can act as a costimulatory molecule during pre-TCR signaling and thus contributes to generation of the DP compartment (21).

However, we subsequently generated CD27-deficient mice that proved to have normal αβ T cell development (12). As reported here, gene expression profiling revealed that transcription of TNF receptor family member HVEM is upregulated in naïve CD27-/- T cells. HVEM is structurally similar to CD27 and expressed on thymocytes. It was reportedly not required for T cell development (24), but we considered the possibility that CD27 and HVEM compensated for each other’s deficiency during thymic T cell development. To examine this, CD27-/-HVEM-/- mice were generated and analysed with regards to T cell development. We found that αβ T cells develop normally in CD27-/-HVEM-/- mice. Moreover, these T cells behave comparably to CD27-/- T cells upon stimulation after influenza virus infection in vivo. We conclude therefore, that HVEM does not compensate for the absence of CD27 during T cell development. Furthermore, we demonstrate that increased HVEM expression on mature CD27-/- CD8+ T cells does not contribute to the reduced accumulation of these cells at priming and tissue sites after influenza virus infection.
RESULTS

T cell development in CD27-deficient mice is normal.

We previously found that injection of anti-CD27 mAb in vivo inhibits generation of the CD4^+8^+ thymic compartment (21). This suggested that CD27 provides a co-stimulatory signal for pre-TCR-induced thymocyte development. However, we subsequently found that CD27 deletion does not affect cellularity and composition of the thymus (12, see also Figure 4). We considered therefore that 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 arrested at the DN2 stage due to lack of pre-TCR expression (20,22). In these mice, no selection has taken place for cells able to make the transition to the DP (CD4^+8^) stage. Wild-type (WT) and CD27^-/- RAG-1-deficient mice were treated with anti-CD3ε mAb, which mimics pre-TCR signalling (23), in the presence or absence of anti-CD27 mAb. After 4 days, thymi were analysed by flow cytometry. As shown in Figure 1, anti-CD3ε 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 CD3ε-induced generation of the CD4^+8^+ compartment in WT, but not in CD27^-/- mice (Figure 1). We conclude that anti-CD27 mAb specifically inhibits pre-TCR induced thymocyte development, but CD27/CD70 interactions are not a prerequisite for pre-TCR signalling to be effective.

HVEM is upregulated on naïve CD27^-/- T cells and thymocytes

To examine potential intrinsic differences between WT and CD27^-/- T cells at the transcriptional level, naïve CD8^+ T cells from WT or CD27^-/- OT-I mice were subjected to genome wide microarray analysis. OT-I mice are transgenic for a TCR that recognizes ovalbumin (OVA) peptide SIINFEKL in the context of H-2K^b (25). Increased mRNA expression of HVEM was observed in naïve CD27^-/- T cells at steady state conditions (Figure 2A). In addition, WT or CD27^-/- OT-I T cells were activated in vitro using artificial APC that present OVA/H-2K^b complex or in vivo by immunizing WT or CD27^-/- OT-I mice with OVA protein (14). Also in these conditions, HVEM mRNA levels were higher in CD27^-/- OT-I T cells than in WT cells (Figure 3A). Furthermore, HVEM mRNA levels were determined in different thymic subsets of naïve WT or CD27^-/- mice.
using quantitative real time PCR. Increased levels of HVEM mRNA were observed in CD27-/- thymocytes as compared to WT at the DN- and CD4+ or CD8+ single positive (SP) stages, but not at the DP stage (Figure 2B). Cell surface expression of HVEM protein was determined by flow cytometry using fluorescently-labeled BTLA tetramers, which is possible since HVEM is the unique ligand for BTLA (26). In the DN populations of the thymus, HVEM cell surface expression was found to be significantly higher on CD27-/- thymocytes than on WT thymocytes at the DN3 and DN4 stages, but not at the DN1 or DN2 stages (Figure 2B). This translated into a significantly higher expression on the total DN population (Figure 3B). In addition, surface expression of HVEM protein was also significantly higher on the CD4+ and CD8+ SP thymic populations of CD27-/- mice as compared to WT (Figure 3B). HVEM expression was increased on CD4+ and CD8+ T cells in the spleens of CD27-/- mice as compared to WT, but was negative on B cells (Figure 3C). In conclusion, HVEM expression is significantly higher in CD27-/- than in WT mice on DN3 thymocytes and on mature CD4+ and CD8+ thymocytes and peripheral T cells.

T cell development is normal in mice lacking both CD27 and HVEM

To explore a possible complementation of CD27-deficiency by HVEM upregulation during thymocyte development, we analysed thymic populations in mice that were single- or double deficient for each molecule. The thymic cellularity of WT, CD27-/-, HVEM-/- and CD27;HVEM-/- mice was examined by flow cytometry (Figure 4A,B). No differences were observed in the sizes of the DN, DP, or SP thymocyte populations between these mouse strains (Figure 4A). In addition, the sizes of the different DN thymocyte populations were examined but revealed no difference

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**Figure 2. HVEM mRNA level is higher in CD27-/- than in WT T cells.**

(A) Micro-array. WT or CD27-/- OT-I T cells were isolated and mRNA was extracted immediately (naive). In addition, OT-I T cells were activated using CD70-expressing artificial APC for the indicated periods of time (in vitro). Also, OT-I T cells were stimulated by intranasal immunization of mice with OVA protein. T cells were isolated on day 3 or 8 from DLN and lung (in vivo). Differences in mRNA expression are indicated as a 2-log ratio of WT versus CD27-/-: A negative ratio indicates a down-regulation in WT OT-I cells. In all measurements indicated P values were below 0.00003. (B) Levels of Hvem mRNA were determined by quantitative real time PCR in DN, DP, or CD4+ or CD8+ SP thymic populations and related to mRNA levels of the household gene Gapdh. Data are means of two independent reactions (+ S.D.).
between these groups (Figure 4B).

In the secondary lymphoid organs, a significant increase in the percentages of CD4+ T cells and a decrease in the percentages of CD8+ T cells was observed in both HVEM-/- and CD27-/-HVEM-/- mice (Figure 5A). The altered percentile seemed primarily attributable to an increase in absolute numbers of CD4+ T cells (Figure 5B). The altered CD4/CD8 ratio as result of HVEM-deficiency was not observed in single positive thymocytes, but was significant in the spleen and inguinal lymph nodes (Figure 5C). This may be a reflection of the process in which HVEM acts as a ligand for the inhibitory receptor BTLA, of which the expression during non-inflammatory conditions is only restricted to B cells in the spleen and lymph nodes (28). In accord with previous analysis (12), percentages and absolute numbers of CD4+ and CD8+ T cells in the spleen and inguinal lymph nodes of CD27-/- mice were in the WT range (Figure 5A,B).

These data indicate that αβ T cell development is normal in mice that lack both CD27 and HVEM. We conclude therefore that HVEM upregulation is not required to compensate for CD27 deficiency during T cell development.

Figure 3. Cell surface expression of HVEM is higher in CD27-/- than in WT T cells.

Surface expression of HVEM protein was determined by flow cytometry using fluorescently labeled BTLA tetramers. WT or CD27-/- cells were examined for HVEM expression gated on the different DN populations in the thymus (A) or gated on the DN, DP or CD4+ or CD8+ SP thymic populations (B). Values are means of 4 samples (+ SEM). T-test indicated significant differences for *P < 0.05. (C) Alternatively, HVEM expression was measured on CD4+ T cells, CD8+ T cells or B cells from the spleen. Shown is a representative sample. MFI = mean fluorescence intensity.
Increased HVEM expression does not affect the phenotype of CD27-/- T cells

To determine whether HVEM upregulation on CD27-/- T cells affected their function, WT, CD27-/- and CD27-/-HVEM-/- mice were analysed for the T cell response to intranasal infection with influenza virus. At successive days after infection, groups of mice were sacrificed and their lung draining lymph nodes (DLN), spleens and lungs were analyzed for the presence of virus-specific CD8+ effector T cells. In agreement with previous results in this model (12-14), the accumulation of total effector CD8+ T cells and those specific for the immunodominant NP366-374 epitope was impaired in the DLN (not shown), spleen and lung of CD27-/- mice (Figure 6A,B). However, both NP-specific and total CD8+ effector T cell numbers in CD27-/-HVEM-/- mice were similar to those in CD27-/- mice in all organs at all time points measured (Figure 6A,B).

Furthermore, cell suspensions from the DLN, spleen and lung were re-stimulated in vitro with NP-peptide and IFNγ and IL-2 expression by CD8+ effector T cells was measured in these cultures. Both IL-2 and IFNγ expression were significantly impaired in CD27-/- CD8+ effector T cells as compared to WT CD8+ effector T cells throughout the response to influenza virus. Importantly, however, no differences in cytokine expression were observed between CD27-/- and CD27-/-HVEM-/- CD8+ effector T cells (Figure 7). In conclusion, the increased surface expression of HVEM on CD27-/- T cells, does not contribute to the previously documented functional phenotype of CD27-/- CD8+ T cells.

Figure 4. Thymic development is normal in absence of CD27 and HVEM.

WT (CD27+/-HVEM+/-), CD27-/- (CD27-/-HVEM+/-), HVEM-/- (CD27+/-HVEM-/-) or CD27-/-HVEM-/- mice were generated and total thymocytes (A) or DN thymocyte subpopulations (B) were analyzed by flow cytometry. Top panels represent the percentage of cells per total thymus (A) or the percentages within the DN populations (B). Lower panels show the corresponding absolute numbers of cells per animal. Values are means of 3 to 9 mice per group (+ SEM).
DISCUSSION

We document here in detail that CD27 is not essential for the intrathymic development of the \( \alpha \beta \) T cell lineage in the mouse. Nevertheless, in CD27\(^{-} \) mice the expression of TNF receptor family member HVEM is upregulated at the DN3 stage of thymic development. This is the exact stage at which in WT mice CD27 is upregulated by pre-TCR signaling and the stage at which anti-CD27 antibody inhibited pre-TCR driven generation of the DP thymocyte compartment (21). We demonstrate here that this inhibitory effect of anti-CD27 antibody on CD3-driven pre-T cell expansion and differentiation in RAG-1\(^{-} \) mice is specific, since the antibody did not have any effect in RAG-1\(^{-} \) mice that were genetically deficient for CD27. However, we also show that CD3 was equally capable to drive the generation of the DP thymocyte compartment in RAG-1\(^{-} \) and CD27\(^{-} \)RAG-1\(^{-} \) mice. Our conclusion from these data was that CD27 plays no role in pre-TCR driven development of the \( \alpha \beta \) T cell lineage in the mouse.

However, we considered that CD27-deficiency was compensated by HVEM when we found HVEM to be upregulated exactly at the DN3 stage of thymocyte development. Therefore, we generated CD27\(^{-} \)HVEM\(^{-} \) mice to address this possibility. However, no defect in generation

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**Figure 5.** Altered CD4/CD8 ratio in secondary lymphoid organs in the absence of HVEM. WT (CD27\(^{+} \)HVEM\(^{+} \)), CD27\(^{-} \) (CD27\(^{-} \)HVEM\(^{+} \)), HVEM\(^{-} \) (CD27\(^{+} \)HVEM\(^{-} \)) or CD27\(^{-} \)HVEM\(^{-} \) mice were sacrificed and CD4\(^{+} \) and CD8\(^{+} \) T cell populations from the spleens and inguinal lymph nodes (LN) were analyzed by flow cytometry. Shown are the percentages (A) or the absolute numbers (B) of CD4\(^{+} \) and CD8\(^{+} \) T cells per organ. (C) In addition, the ratio of CD4\(^{+} \) versus CD8\(^{+} \) T cells is determined for the thymus, spleen and LN. Values are means of 3 to 9 mice per group (+ SEM). T-test indicated significant differences for *\( P < 0.05 \) and **\( P < 0.01 \).
of the DP thymocyte compartment was found in these mice. It was also striking that in CD27−/− mice, HVEM was additionally upregulated at the SP mature thymocyte stage that is driven by TCRαβ-mediated positive selection (20). Nevertheless, intrathymic development of the αβ lineage was completely normal in mice deficient for both CD27 and HVEM. Two possibilities remain: either CD27 does not play a role in thymic development of conventional αβ T cells

Figure 6. Increased HVEM expression in CD27− T cells does not contribute to impaired CD8+ effector T cell accumulation.

WT (CD27+/HVEM+/), CD27− (CD27−HVEM+/) or CD27−HVEM− mice were infected with influenza virus and DLN, spleen and lung were isolated at day 6, 8, 10 or 14 after infection. (A) Representative FACS plots of NP tetramer and CD62L staining in the spleen and lung at day 8 and 10 after infection. Numbers represent the average percentages of NP tetramer+ and CD62Llow T cells per organ. Values are means of 4 mice per group (+ ST.DEV). (B) Absolute numbers of CD62Llow CD8+ T cells (CD8+ Teff) (top panel), or NP tetramer+ CD8+ T cells (lower panel) per organ. Values are means of 4 mice per group (+ SEM). T-test indicated significant differences for *P < 0.05 and **P < 0.01.
in the mouse, or the redundancy between CD27 and other TNF receptor family members in this respect is not restricted to HVEM. In fact, preliminary gene array experiments revealed a modest induction of OX40 mRNA in CD27⁻/⁻HVEM⁻/⁻ DN3 cells (data not shown).

Importantly, the observation of increased HVEM surface expression on naive CD27⁻/⁻ T cells in the periphery prompted us to investigate the consequences thereof. Besides being a receptor for TNF family member LIGHT, HVEM is also a ligand for the inhibitory receptor BTLA that is expressed on B cells and activated Tₚ1 cells in the periphery (28). Interpretation of the consequences of HVEM deficiency is complex, since the molecule might have both positive and negative effects on the T cell response, depending on the engagement of either LIGHT or BTLA (24,26,28,30). It has been reported that the HVEM-LIGHT interaction is dispensible for the CD8⁺ T cell response (30). However, given the HVEM upregulation on CD27⁻/⁻ T cells, we considered that HVEM-BTLA interaction might contribute to the T cell phenotype observed in CD27⁻/⁻ mice.

Previously, we have shown that CD27 contributes to clonal expansion and maintenance of CD8⁺ effector T cells during an influenza virus infection by using CD27⁻/⁻ mice. We specified that CD27 rescues clonally expanding CD8⁺ T cell from

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**Figure 7.** Increased HVEM expression in CD27⁻/⁻ T cells does not contribute to impaired cytokine expression of CD8⁺ effector T cells.

WT (CD27⁺/⁺HVEM⁺/⁺), CD27⁻/⁻ (CD27⁻/⁺HVEM⁺/⁻) or CD27⁻/⁻HVEM⁻/⁻ mice were infected with influenza virus and DLN, spleen and lung were isolated at day 6, 8, 10 or 14 after infection. Total suspension cells extracted from the indicated organs were re-stimulated for 4 h in vitro with NP366-374 peptide, stained for IFN-γ, IL-2 and CD8 and analysed by flow cytometry. The average percentages of IFN⁺ (top panel) or IL-2⁺ (lower panel) cells within the CD8⁺CD62L⁺⁺ (CD8⁺ Teff) are shown. Values are means of 4 mice per group (+ SEM). T-test indicated significant differences for *P < 0.05 and **P < 0.01.
apoptosis, but did not detectably affect cell cycle activity (12,13). In addition, we showed that CD27-CD70 interaction promotes the formation of CD8+ memory T cells (31). These data were confirmed by reverse approaches, in which WT CD8+ T cell responses were examined and soluble recombinant CD70 was infused, CD27-CD70 signaling was blocked by inhibitory anti-CD70 mAb, or CD70 was expressed transgenically on B cells or DC (32-37). In agreement with this, we now find that despite the upregulation of HVEM on CD8+ T cells, the quantitative response of CD27− CD8+ T cells to influenza virus infection is not different from that of CD27−HVEM− CD8+ T cells. Therefore, we conclude that HVEM upregulation on CD27− CD8+ T cells is not responsible for (part of) the previously reported phenotypes of CD27− CD8+ T cells.

We identified CD27-directed gene products in CD8+ T cells by using a variety of approaches, in which either responder T cells were WT or CD27-deficient, or responder T cells were WT and CD70 expression on the APC was the variable. We considered only those genes that were altered in expression under both conditions as significant. Therefore, all genes that were identified in CD8+ T cells as direct or indirect CD27 targets are indeed CD27-regulated and not expressed as a consequence of HVEM engagement. We identified IL-2 as a very important CD27-regulated gene product and recently showed that CD27-mediated survival of influenza virus-specific CD8+ effector T cells in infected tissue is regulated by autocrine IL-2 signaling (14). During clonal expansion in vivo, another mechanism is responsible for CD27-mediated T cell survival that impinges on Bcl-xL and the Pim kinase pathway (this thesis).

By using B-cell specific CD70 transgenic mice, Arens and colleagues have shown that CD27 signaling promoted IFNγ expression by CD8+ T cells (38). Others have shown that ConA stimulated HVEM− CD8+ T cells have increased expression of both IL-2 and IFNγ (24). This increased cytokine expression was not due to impaired binding of HVEM to its survival promoting ligand LIGHT, since LIGHT− CD8+ T cells showed a normal cytokine expression pattern. Increased cytokine expression was dependent on the presence of CD4+ T cells, since removal of CD4+ T cells returned it back to WT levels (24). Most likely, HVEM binds to BTLA on CD4+ T cells and inhibits the expression of both IL-2 and IFNγ in this model. In line with this, it has been shown that crosslinking of BTLA reduced T cell specific IL-2 expression (28). Based on these data, it seemed conceivable that increased levels of HVEM on CD27− T cells would lead to a reduced level of IL-2 and IFNγ expression. We show here that absence of CD27 expression does result in a significant decrease in the capacity of CD8+ effector T cells to express IL-2 and IFNγ in priming organs and in the infected tissue. Importantly, however, detailed analysis of CD27−HVEM− mice revealed no contribution of HVEM to the CD27− CD8+ T cell phenotype in this model of influenza virus infection. This was further confirmed by analysis of the response in HVEM− mice, which was no different from that in WT mice (data not shown). It appears therefore that HVEM is dispensable for the CD8+ T cell response to influenza virus in mice.

Since BTLA is primarily expressed on B cells and activated Tγ1 cells (28), an impact of increased HVEM expression on CD27− T cells might be reflected in models that depend more on the action of B cells and Tγ1 cells. With regards to the CD4+ T cell response, we have primarily used CD27− mice to determine the effects of CD27-CD70 interaction. However, the phenotypes of CD27− CD4+ T cells we have described thus far (27) are in agreement with the results obtained with anti-CD70 blocking in WT mice and the use of CD70 transgenic APC (38,39). Preliminary analysis of the CD4+ T cell response to influenza virus as performed in the current study revealed no important contribution of HVEM on CD27−
CD27 and T cell development

CD4+ T cells (results not shown). However, in future studies where the effects of CD27 stimulation are examined, it will remain important to complement the analysis of CD27−/− T cells with conditions of CD70 stimulation, blocking or deficiency. Since we have generated CD70-deficient mice, and have agonistic soluble recombinant CD70 and CD70 blocking antibody available, these studies can be adequately performed.

MATERIAL AND METHODS

Mice. WT, CD27−/− (12), HVEM−/− (24), CD27−/−HVEM−/−, OT-I (25), OT-I;CD27−/−, RAG-1−/− (22) and CD27−/−RAG-1−/− mice on a C57BL/6 background were used for experiments at 7-12 weeks of age. Experiments were approved by the Experimental Animal Committee of The Netherlands Cancer Institute (DEC) and performed in accordance with national guidelines.

Flow cytometry. Cells were isolated from lungs, spleens and DLN and stained with fluorochrome-conjugated antibodies, BTLA tetramers or MHC tetramers as described (27). Antibodies used were anti-CD8β (53-6.7), anti-CD4 (L3T4), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD19 (1D3), anti-CD3ε (145-2C11), anti-CD27 (LG.3A10), anti-IL-2 (XMG1.2), and anti-CD62L (MEL-14). These mAb were obtained from BD Biosciences, eBioscience Inc. (San Diego CA), or prepared as purified Ig from available hybridomas. Allophycocyanin-labeled BTLA- and H-2Dβ/NP366-374 tetramers were prepared as described (28). Cells were analyzed using a FACSCalibur (BD, Franklin Lakes NJ) or CyAn (Dako, Glostrup, Denmark) flow cytometer. Data were analyzed with FCS Express (De Novo Software, Los Angeles CA) or FlowJo (Tree Star Inc., Ashland OR) analysis software. Propidium iodide (PI)-stained dead cells were excluded from analysis. T cells used for microarray or real time PCR were sorted by flow cytometry (FACSria, BD) and kept on ice immediately after sorting. For ex vivo intracellular staining, single cell suspensions were stimulated by culture with with NP366-374 Peptide (sequence: ASNENMDAM, 100 μg/ml) for 4 h, in the presence of recombinant human IL-2 (40 U/ml, Chiron) and GolgiPlug (1 μl/ml, BD Biosciences). After incubation, cells were surface stained for CD8 and CD62L for 30 min on ice, washed, incubated in Cytofix/Cytoperm solution (BD Biosciences) for 20 min on ice, washed and stained for IL-2 and IFN-γ on ice for 30 min.

T cell purification. For in vitro cultures, T cells were purified from spleens of OT-I mice as described (27). Purity of the resulting T cell populations was checked by flow cytometry using anti-TCR Vβ5.1/5.2 and anti-CD8 mAbs and was always greater than 90%. For microarray purposes, T cells were purified after co-culture with artificial APC with CD8 MACS beads according to manufacturer’s protocol (Miltenyi Biotec). Purity of the resulting T cell populations was always greater than 98%.

Cell culture. For in vitro priming of OT-I T cells, the mouse fibroblast line MEC.B7.SigOVA was used that expresses a minigene encoding the OVA257-264 epitope, which is presented in the context of H-2Kb. These aAPC were available in two versions that did or did not express CD80 (29). They were retrovirally transduced to stably express mouse CD70 and GFP from a bicistronic construct and flow cytometrically sorted to isolate lines with highly comparable levels of H-2Kb expression. Cells were seeded at 10^5 per well in 24-well plates and cultured overnight to form an adherent monolayer. The next day, wells were washed with medium to remove any non-adherent cells or cell debris. WT or CD27−/− OT-I T cells that had been labeled with CFSE (5 μM), following the manufacturer’s protocol (Invitrogen, Molecular Probes) were added at 0.5 x 10^6 per well.
in culture medium and plates were centrifuged at 900 g for 1 min. After 20 h of co-culture, the non-adherent T cells were gently transferred to empty wells and cultured further. At the indicated time points after initial stimulation with the artificial APC, T cell numbers were determined using a CASY cell counter (Scharfe System GmbH). PI or TO-PRO-3 was added and cell division and death were monitored by flow cytometry.

**In vivo treatments**

CD27-/- mice were bred with RAG-1-/- mice. Mice that were RAG-1-/- 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. Mice were infected intranasally with 25 hemagglutinin units of influenza virus strain A/NT/60/68 as described (12-14). Alternatively, mice were immunized intranasally with 500 μg OVA protein plus 1 μg cholera toxin (Sigma) in 50 μl HBSS (27).

**Gene expression profiling.** RNA extraction, amplification and hybridization were performed as described (27). Microarrays spotted with the Operon v3 oligonucleotide library were obtained from the central microarray facility of The Netherlands Cancer Institute (http://microarrays.nki.nl). Microarrays were scanned on Agilent Technologies scanner and data extraction was done using Imagene 6.0 software (BioDiscovery, El Segundo, CA). Each experiment consisted of two microarrays, to allow for dye reversal between the samples, thus reducing systemic errors due to oligonucleotide-specific dye preferences. The heat map in Figure 2a was created using TIGR (The Institute for Genomic Research) MultiExperiment Viewer software, version 3.1. Genes were included in the analysis when found to be differentially expressed with a p-value below 0.00003 in at least two different experimental set-ups. Hierarchy in this list was determined by the fold differential expression (M value) and the number of experimental set-ups in which the gene was found to be differentially expressed. The p-value is based on various parameters, including signal intensity (A value), ratio of representation (M value) and signal quality.

**Statistical analysis.** Statistical significance was determined using a 2 tailed Student’s t test. Differences were found to be significant when $P < 0.05$.

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