CD27 is required for generation and long-term maintenance of T cell immunity

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The Traf-linked tumor necrosis factor receptor family member CD27 is known as a T cell costimulatory molecule. We generated CD27$^{-/-}$ mice and found that CD27 makes essential contributions to mature CD4$^+$ and CD8$^+$ T cell function: CD27 supported antigen-specific expansion (but not effector cell maturation) of naïve T cells, independent of the cell cycle–promoting activities of CD28 and interleukin 2. Primary CD4$^+$ and CD8$^+$ T cell responses to influenza virus were impaired in CD27$^{-/-}$ mice. Effects of deleting the gene encoding CD27 were most profound on T cell memory, reflected by delayed response kinetics and reduction of CD8$^+$ virus-specific T cell numbers to the level seen in the primary response. This demonstrates the requirement for a costimulatory receptor in the generation of T cell memory.

The tumor necrosis factor (TNF) receptor family contains death receptors, as well as receptors that bind TNF receptor–associated factors (Trafs)[1]. Many TNF receptor family members and their TNF-related transmembrane ligands are expressed on cells of the immune system where they play important roles. The Traf-linked receptor subgroup includes CD27 (also known as TNFRSF7), CD30, OX-40 (also known as CD134) and 4-1BB (also known as CD137), which selectively regulate lymphocyte function[2]. Other prominent members are RANK and CD40, which additionally modulate the response of dendritic cells (DCs)[3,4]. Traf-linked receptors have been implicated in cell proliferation, differentiation, survival and migration. For some receptors such as CD30, a death-inducing capacity has been observed, which may be conveyed by membrane TNF[5]. Trafs signal to NF-κB and Jun kinase, but the array of genes they target is undefined. It is proposed that Trafs counteract apoptosis[6-8] *via* the inhibitor of apoptosis proteins (IAPs)[9] and/or NF-κB[10].

The relevance of Traf-linked receptors was initially ascertained by analyzing their expression patterns, as well as their functional effects *in vitro*. CD27, CD30, 4-1BB and OX-40 are exclusively expressed on cells of the lymphoid lineage, mostly in an activation-specific manner, and all enhance T cell receptor (TCR)-induced T cell expansion. CD27 and its ligand, CD70, have been defined at the protein, cDNA and genomic level in both human and mouse[11–16]. CD27 is found on NK, T and B cell populations[17]. In humans and mice, the majority of CD4$^+$ and CD8$^+$ naïve peripheral T cells express CD27 and expression is up-regulated upon TCR stimulation[18–20]. Loss of CD27 expression is irreversible and seems to represent terminal effector T cell differentiation[19,21]. In humans, naïve B cells lack CD27 but antigen receptor stimulation induces expression. CD27$^+$ B cells display all the functional and phenotypic characteristics of memory cells[22,23]. Whether the same is true in mice remains to be shown. Expression of the ligands for CD27 and related receptors is very restricted and tightly regulated by antigen and cytokines[2,17,24,25]. The CD27 ligand CD70 is transiently up-regulated by antigen receptor stimulation on both T and B cells and thus reflects recent antigenic priming[16,17]. CD70 has also been found on thymic DCs in humans[26] and on lymph node DCs from infected mice[27], but not on any other cell types.

CD27 ligation enhances TCR-induced expansion of both CD4$^+$ and CD8$^+$ peripheral T cells, but the nature of this costimulatory signal is ill-defined[13,14,20,28,29]. One study documents that CD27-CD70 interaction promotes differentiation of CD8$^+$ T cells to effector cytotoxic T cells (CTLs)[30]. On B cells, CD27 ligation only marginally enhances expansion, but promotes plasma cell differentiation and immunoglobulin production[31]. The CD27-CD70 pair may contribute to T cell expansion through T–T cell, and possibly T cell–DC, interactions and to B cell differentiation through T–B cell and/or B–B cell interactions. Targeted deletion of receptors and ligands in mice is the optimal approach to determine the exact contribution each Traf-linked TNF receptor family member makes to the immune system. We present here the effects of targeting of the gene encoding CD27 (*Tnfrsf7*) on T cell development and peripheral responsiveness. Our analysis revealed that CD27 is required for effective generation and long-term maintenance of antigen-specific CD4$^+$ and CD8$^+$ T cell immunity.

Results

Generation of CD27-deficient mice

Genomic clone λ5, containing the complete coding region of *Tnfrsf7*, was isolated and characterized by Southern blotting and nucleotide sequencing (**Fig. 1a**). This led to the definite localization

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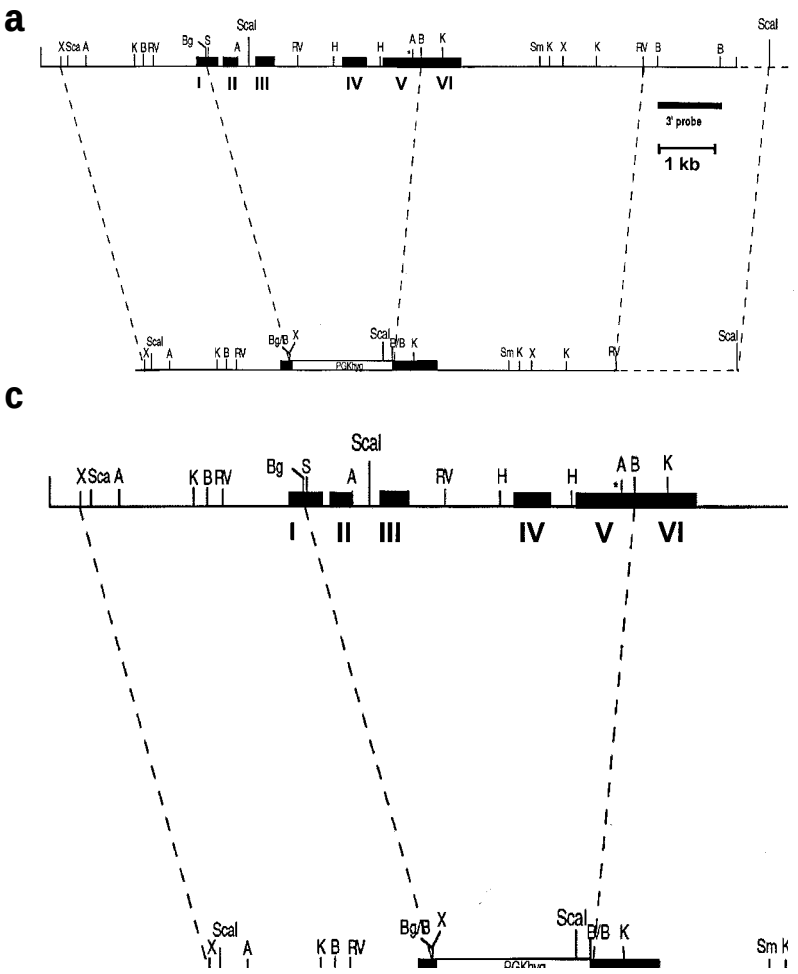

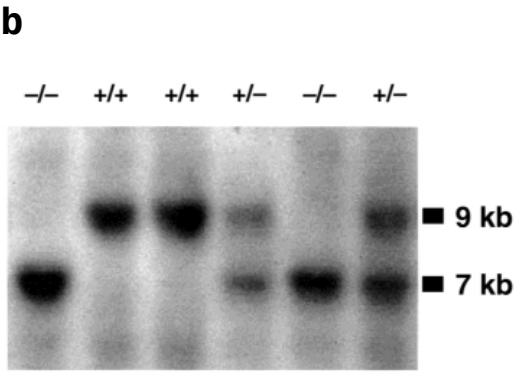

Figure 1. *Tnfrsf7*-targeting strategy and result. (**a**) Restriction map of the λ5 clone encoding murine *Tnfrsf7* and the targeting construct derived from it. The dashed line indicates genomic DNA located outside of λ5. Restriction sites indicated are: A: *Apa*1, B: *Bam*HI, Bg: *Bgl*II, H: *Hind*III, K: *Kpn*I, RV: *Eco*RV, S: *Sph*I, *Sca*I, Sm: *Sma*I and X: *Xba*I. Exons are depicted as wide filled bars (I–VI). The open reading frame of mCD27 starts between *Bgl*II and *Sph*I sites and ends at the asterix (*). (**b**) Southern blot of mouse-tail DNA cut with *Sca*I and probed with the 3′ probe indicated in **a**, visualizing a 9-kb WT *Tnfrsf7* fragment and a 7-kb mutated *Tnfrsf7* fragment. (**c**) Flow cytometric analysis of spleen cells from $CD27^{+/+}$ mice and $CD27^{-/-}$ mice. Cells were double stained as indicated.

of exons I, II, V and VI. Exons V and VI are separated by an intron of 30 nucleotides and contain the end of the coding region and the 3′ untranslated region. Exons III and IV were not localized exactly but are contained in the indicated restriction fragments. In the targeting construct, the complete *Tnfrsf7* coding region was replaced by the hygromycin-resistance gene. After selection, DNA from transfected embryonic stem (ES) cell clones was cut with *ScaI* and hybridized with the 3′ probe (**Fig. 1a**). Homologous recombination yielded a hybridizing fragment of 7 kb, whereas the wild-type (WT) fragment is 9 kb. A homologous recombinant ES cell clone was injected into C57BL/6 blastocysts. Resulting chimeric mice were crossed with FVB mice to attain germ line transmission of the mutated *Tnfrsf7* allele (**Fig. 1b**). Immunofluorescence analysis of spleen cells showed expression of CD27 in WT mice on the great majority of both $CD4^+$ and $CD8^+$ T cell subsets and a small proportion of B cells, as reported previously[20]. It also confirmed the CD27-deficient phenotype (**Fig. 1c**). $CD27^{-/-}$ mice proved viable, fertile and remained healthy over prolonged periods of time (>6 months).

T cell division upon TCR stimulation

Mice were backcrossed seven or more times to C57BL/6 and 129/OLA backgrounds. In all experiments, $CD27^{+/+}$ and $CD27^{-/-}$ littermates were compared. $CD27^{+/+}$, $CD27^{+/-}$ and $CD27^{-/-}$ mice did not differ in bone marrow, thymus, spleen or lymph node cellularity. Also, the T or B cell, and $CD4^+$ or $CD8^+$ subset, composition of lymphoid organs was normal in $CD27^{-/-}$ mice. In the thymus, the size of $CD4^-CD8^-$, $CD4^+CD8^+$ and mature $CD4^+CD8^-$ and $CD4^-CD8^+$ populations, as well as subset composition of the $CD4^-CD8^-$ compartment were similar to WT (data not shown). Thus, CD27 was not required for antigen-independent expansion and differentiation of (precursor) T and B cells or for long-term survival of naïve lymphocytes.

Studies have implicated CD27 in costimulation of peripheral $CD4^+$ and $CD8^+$ T cells: TCR-induced [^{3}H]thymidine incorporation is enhanced by monoclonal antibody (mAb) to CD27 or recombinant ligand[13,14,20,28,29]. Conversely, mAbs to CD70 inhibit this response, indicating that CD27-CD70 interaction supports TCR-induced T cell expansion[16,29]. Purified, naïve peripheral T cells from $CD27^{-/-}$ mice showed significantly decreased TCR-induced [^{3}H]thymidine incorporation as compared to WT T cells: 1.8-fold over a concentration range of mAb to CD3ε (**Fig. 2a**). The same result was obtained when incorporation of radioactivity was assayed after 1 or 2 days of culture (data not shown). Recombinant interleukin 2 (IL-2) or costimulation with a mAb to CD28 increased the expansion of both WT and $CD27^{-/-}$ cells, but did not rescue the defect in $CD27^{-/-}$ cells (**Fig. 2a**). Thus, reduced [^{3}H]thymidine incorporation was not caused by deficient IL-2 production and the CD27 costimulatory signal was qualitatively different from the CD28 signal.

The cell-permeant fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) was used to study the effect of CD27 on cell cycle activity. Upon cell division, CFSE is equally distributed between daughter cells, allowing resolution of up to eight cycles of cell division by flow cytometry. Costimulation *via* CD28[32] increased the proportion of T cells entering into cell cycle on TCR stimulation, as well as cell cycle activity (number of cycles completed within a 3-day time period) (**Fig. 2b**). However, absence of CD27 did not affect either cell cycle entry or activity (**Fig. 2b**). Similarly, costimulation of CD3-activated WT T cells with mAb to CD27 did

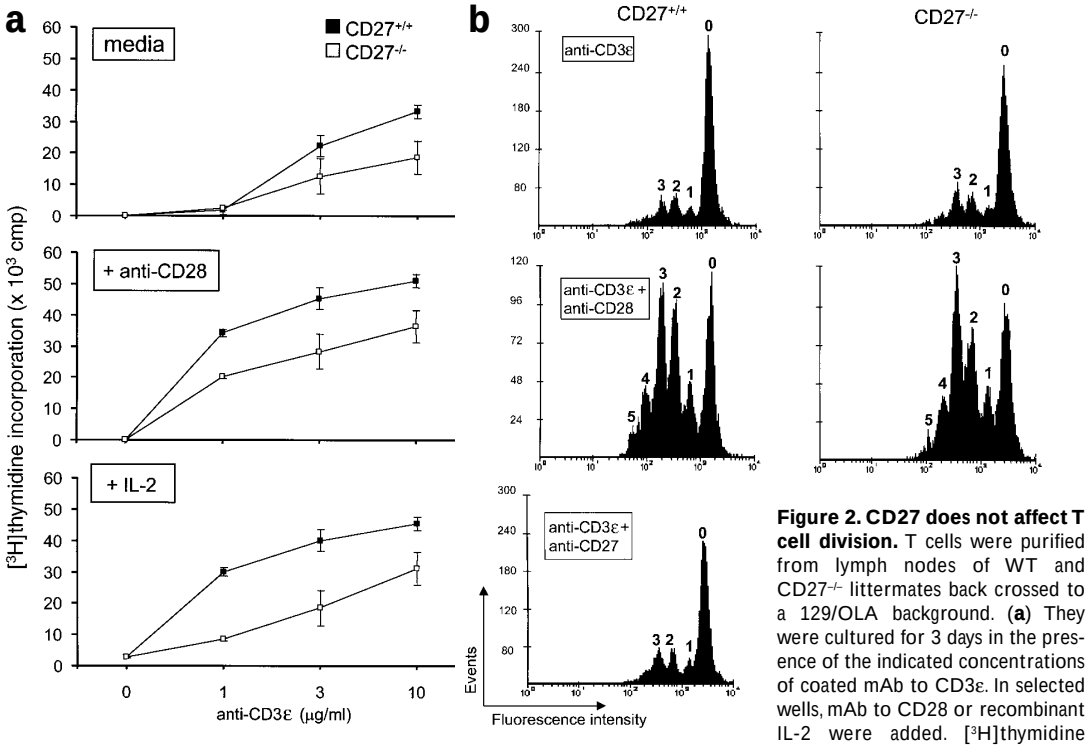

Figure 2. CD27 does not affect T cell division. T cells were purified from lymph nodes of WT and CD27$^{-/-}$ littermates back crossed to a 129/OLA background. (**a**) They were cultured for 3 days in the presence of the indicated concentrations of coated mAb to CD3ε. In selected wells, mAb to CD28 or recombinant IL-2 were added. [^{3}H]thymidine incorporation proceeded for 8 h at the end of the culture period. (Values are the mean±s.d. of triplicate cultures in one experiment. The experiment was repeated four times with similar results.) (**b**) Purified T cells were labeled with the fluorescent dye CFSE before incubation in the presence of coated mAb to CD3ε and mAb to CD28, or CD27 as indicated. After 3 days, cells were collected and analyzed by FACS after gating of live cells.

not influence cell division. The CFSE-staining pattern obtained was identical to the pattern obtained after stimulation with mAb to CD3 alone (**Fig. 2b**). Lack of effect of mAb to CD27 cannot be explained by lack of CD27 expression: the great majority of naive peripheral T cells express CD27 (**Fig. 1c**) and costimulation with mAb to CD27 enhances [^{3}H]thymidine incorporation three- to fourfold[20]. We conclude that, unlike CD28, CD27 does not influence the cell division process. Its effect on [^{3}H]thymidine incorporation might be explained by an effect on cell survival.

Primary T cell response in CD27$^{-/-}$ mice

To test the capacity of the immune system to mount an antigen-specific T cell response in the absence of CD27-CD70 interaction, we studied influenza virus infection in C57BL/6 mice. Major histocompatibility complex (MHC) H2-D^b tetramers loaded with the immunodominant influenza NP(366–374) peptide allow visualization and quantification of virus-specific CD8$^+$ T cells[33,34]. Mice were infected intranasally with 25 hemagglutinin units (HAU) of influenza virus A/NT/60/68. At days 6, 8, 10 and 14 after infection, absolute numbers of lung-infiltrating CD4$^+$ and CD8$^+$ T cells were measured (**Fig. 3a**). In WT mice, T cell numbers were at a maximum 8–10 days after infection, with CD8$^+$ cells outnumbering CD4$^+$ cells. In CD27$^{-/-}$ mice, the overall response curves were lower than in WT mice, for both CD4$^+$ T cells (P=0.014) and CD8$^+$ T cells (P=0.020).

We determined the percentage and absolute numbers of virus-specific CD8$^+$ T cells in lung and spleen using MHC class I tetramers. A representative tetramer *versus* CD8 staining of lung-infiltrating cells is shown in **Fig. 3b**. In the upper panels of **Fig. 3c**, tetramer-positive T cells are plotted as percentage of total CD8$^+$ T cells. At the peak of the response (days 8–10) in WT mice, 10–15% of CD8$^+$ T cells in the lung and 4–5% of CD8$^+$ T cells in the spleen were specific for the immunodominant influenza virus peptide. This translated into 20–30×10^4 virus-specific T cells in the lung and around 6×10^5 virus-specific T cells in the spleen. In lung, a difference in responsiveness between WT and CD27-deficient mice was not apparent from the percentage of tetramer-positive T cells, but it became evident when absolute numbers were calculated based on the size of the infiltrate (**Fig. 3c**, lower panels). The curve for absolute numbers of virus-specific CD27$^{-/-}$ T cells shifted downwards, compared to the curve for CD27$^{+/+}$ T cells, for both lung (P=0.0046) and spleen (P=0.014). Thus, CD27$^{-/-}$ mice displayed a decrease in absolute numbers of CD8$^+$ and CD4$^+$ T cells that infiltrate the lung in response to primary infection and a reduction in absolute numbers of virus-specific CD8$^+$ T cells in lung and spleen, particularly at the peak of the response.

T cell memory in the absence of CD27

To analyze the memory response, CD27$^{-/-}$ and WT mice were challenged with 200 HAU of influenza virus 6 weeks after the first infection. In the lungs of WT mice, the secondary virus-specific T cell response could be measured earlier than the primary, with the peak of the response at day 5 (**Fig. 4a**). Moreover, the response was more pronounced with around 40% of CD8$^+$ T cells (60×10^4 cells) in the lung being virus-specific at the peak (**Fig. 4b**). Quantification of total CD4$^+$ and CD8$^+$ T cells present in the lungs at days 3, 5, 7 and 11 after reinfection revealed that the response was reduced in CD27$^{-/-}$ mice (**Fig. 4a**). For CD4$^+$ T cells, response kinetics in CD27$^{-/-}$ mice were delayed (P=0.053) as compared to WT mice, with a peak at day 7 instead of day 5. At all time points, lung-infiltrating CD4$^+$ T cell numbers in CD27$^{-/-}$ mice were below those found in WT mice. Also for CD8$^+$ T cells, the kinetics of the response were markedly delayed in CD27$^{-/-}$ mice (P=0.0007), with the peak of the response at day 7 instead of at day 5. The curve was also shifted downwards (P<0.0001): CD8$^+$ T cell numbers in lungs of CD27$^{-/-}$ mice were below those in WT mice at days 3 and 5 and peak values remained at the level seen in the primary response (**Fig. 3a**).

The discrepancy in responsiveness of WT and CD27$^{-/-}$ mice could

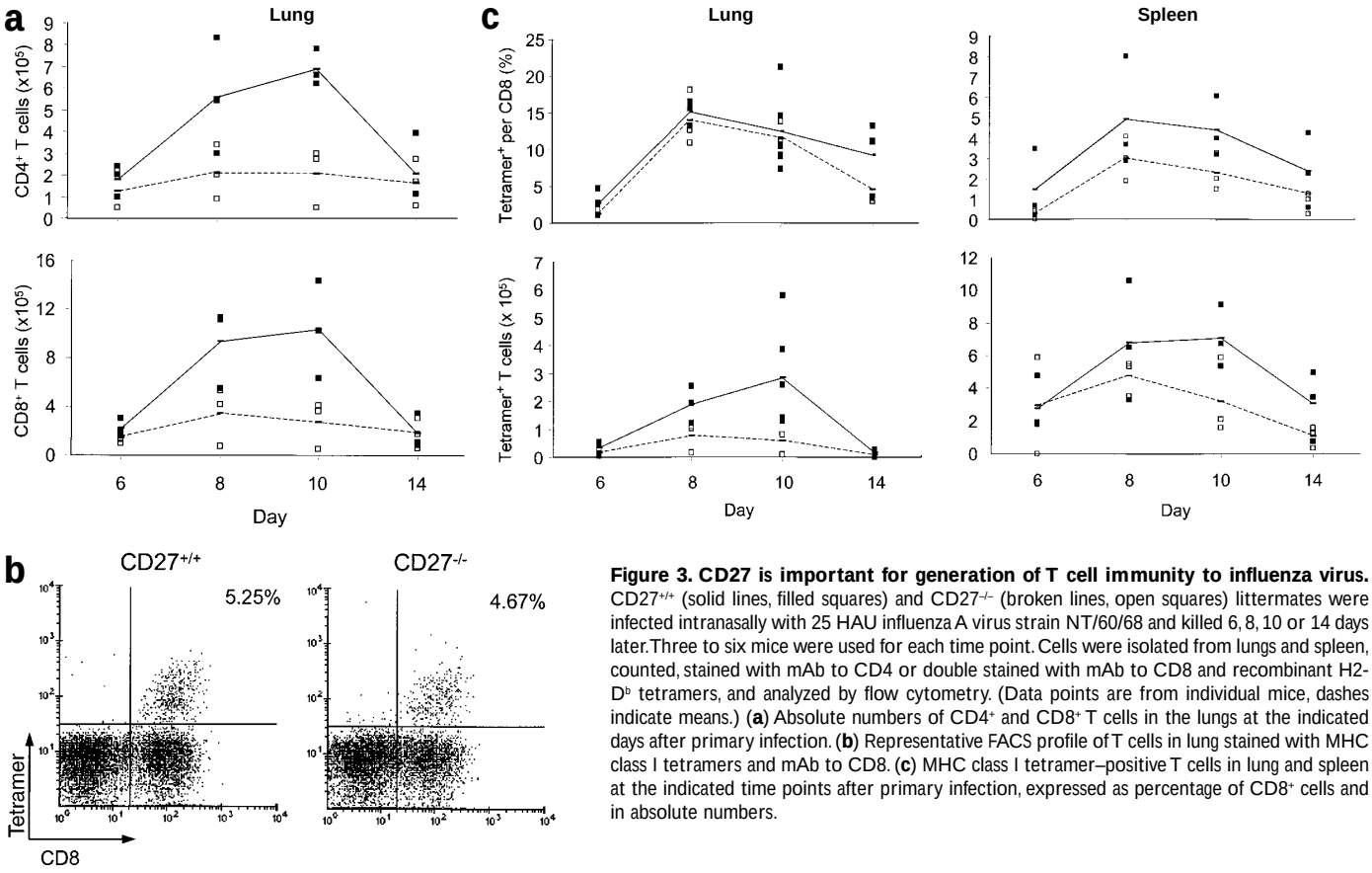

Figure 3. CD27 is important for generation of T cell immunity to influenza virus. $CD27^{+/+}$ (solid lines, filled squares) and $CD27^{-/-}$ (broken lines, open squares) littermates were infected intranasally with 25 HAU influenza A virus strain NT/60/68 and killed 6, 8, 10 or 14 days later. Three to six mice were used for each time point. Cells were isolated from lungs and spleen, counted, stained with mAb to CD4 or double stained with mAb to CD8 and recombinant H2-D^b tetramers, and analyzed by flow cytometry. (Data points are from individual mice, dashes indicate means.) (**a**) Absolute numbers of $CD4^+$ and $CD8^+$ T cells in the lungs at the indicated days after primary infection. (**b**) Representative FACS profile of T cells in lung stained with MHC class I tetramers and mAb to CD8. (**c**) MHC class I tetramer–positive T cells in lung and spleen at the indicated time points after primary infection, expressed as percentage of $CD8^+$ cells and in absolute numbers.

also be seen in the virus-specific $CD8^+$ T cell infiltrate of the lung (**Fig. 4b**). As for the primary response, the percentage of tetramer-positive T cells did not indicate a major difference in responsiveness between WT and CD27-deficient mice. However, when absolute numbers were calculated based on the size of the lung infiltrate, a vast discrepancy became apparent. At day 5, a peak response was found in WT mice, which was lacking in $CD27^{-/-}$ mice (P=0.0003). The response in $CD27^{-/-}$ mice was comparable to the

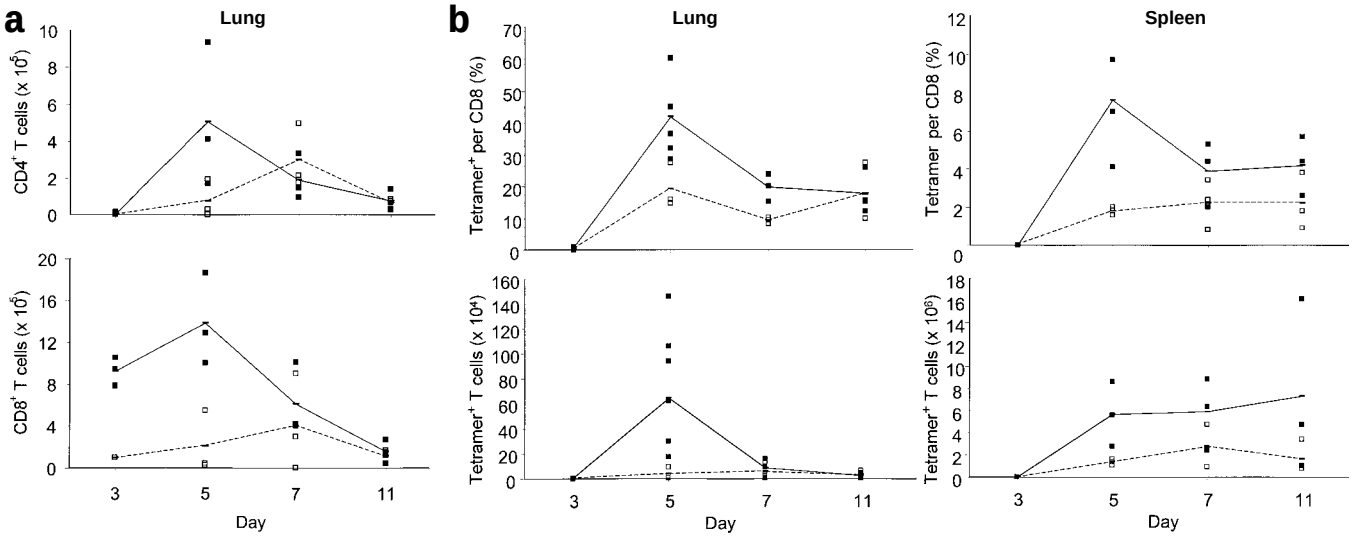

Figure 4. CD27 is required for T cell memory. Six weeks after primary infection, $CD27^{+/+}$ (solid lines, filled squares) and $CD27^{-/-}$ (dashed lines, open squares) were infected intranasally with 200 HAU influenza A virus and killed 3, 5, 7 or 11 days later. (Three to six mice were used for each time point. Cells were isolated and stained as described for Fig. 3. Data points are from individual mice, dashes indicate means.) (**a**) Absolute numbers of $CD4^+$ and $CD8^+$ T cells in the lungs at the indicated days after secondary infection. (**b**) MHC class I tetramer–positive T cells in lung and spleen at the indicated time points after secondary infection, expressed as percentage of $CD8^+$ cells and in absolute numbers.

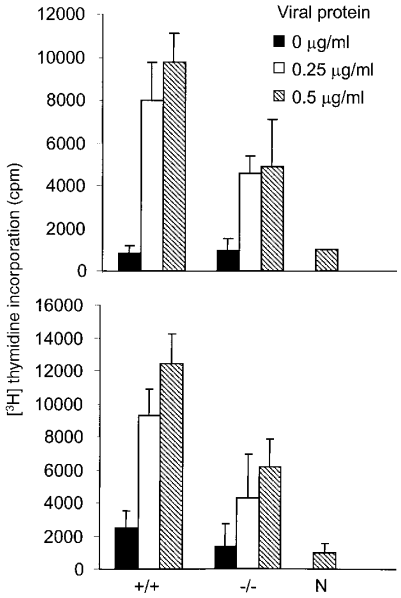

Figure 5. CD27 contributes to the antigen-specific CD4⁺ T cell response. Expansion of splenic CD4⁺ T cells upon challenge with purified, inactivated influenza virus *in vitro*. CD4⁺ T cells were purified and cultured for 4 days with the indicated concentrations of virus, presented by C57BL/6 spleen cells. [³H]thymidine incorporation occurred during the last 8 h of culture. (Values represent the mean ±s.d. of triplicate samples in one experiment. The experiment is representative of three.) T cells were taken from immunized CD27$^{+/+}$ or CD27$^{-/-}$ mice or from nonimmunized (N) WT mice at day 14 after primary infection (upper panel) or day 11 after secondary infection (lower panel).

primary response in terms of percentage (about 15%) and absolute number (about 7×10^4) of tetramer-positive CD8⁺ T cells at the peak. In the spleen, the curve for CD27$^{-/-}$ mice was shifted downwards compared to the curve for WT mice (P=0.0022). In conclusion, the memory T cell response proceeded with slower kinetics in CD27$^{-/-}$ mice and was greatly reduced in terms of absolute numbers of participating CD8⁺ T cells, particularly in the lung.

Virus-specific CD4⁺ T cell responses

Absolute numbers of CD4⁺ T cells infiltrating the lung after primary and secondary virus infection were reduced in CD27$^{-/-}$ mice. To examine whether the antigen-specific CD4⁺ T cell response was affected, we assayed for virus-specific CD4⁺ T cell expansion *in vitro*. Purified CD4⁺ T cells from both CD27$^{+/+}$ and CD27$^{-/-}$ mice showed a specific expansion, dependent on the concentration of viral antigen (**Fig. 5**). The response of CD27$^{-/-}$ CD4⁺ T cells was reduced as compared to the response of WT cells, both after primary and secondary infection, over a wide concentration range of viral protein. This supported the *in vivo* response data, indicating that both primary and memory CD4⁺ T cell responses were affected in CD27$^{-/-}$ mice.

Table 1. CD27 does not affect differentiation towards IFN-γ production

		CD8⁺	CD4⁺
Naïve	+/+	8.2±1.5[1]	11.5±5.0
	–/–	5.9±0.3	9.4±0.1
Primary	+/+	12.2±5.2	7.2±3.6
	–/–	10.8±3.2	10.3±7.5
Secondary	+/+	19.9±8.2	18.3±9.7
	–/–	20.7±11.3	18.8±13.2

Lung-infiltrating cells were stimulated with PMA and ionomycin. IFN-γ–positive cells were quantified within gated CD8⁺ or CD4⁺ cell populations. Results are percentages expressed as mean±s.d. derived from three independent experiments with two mice each per group.

CD8⁺ T cell differentiation into effector cells

To examine whether CD27 signaling contributed to differentiation of peripheral T cells into effector cells, the capacity of CD27$^{-/-}$ T cells to exert cytolysis and to produce interferon γ (IFN-γ) were tested. For cytotoxicity assays, splenocytes from immunized mice were cultured *in vitro* with NP(366–374) peptide and IL-2 to allow the peptide-specific CD8⁺ T cell population to expand. A similar amount of virus-specific CD8⁺ T cells from WT and CD27$^{-/-}$ mice were used. T cells from CD27$^{-/-}$ mice lysed their targets as efficiently as WT controls, after both primary or secondary infection (see Web Figure 1 on the supplementary information page of *Nature Immunology* online). Thus, a lack of CD27 does not affect the differentiation program that shapes cytolytic T cell function.

The capacity to produce IFN-γ was studied in both CD4⁺ and CD8⁺ T cells isolated from mice immunized 8 days previously with influenza virus. Total lung-infiltrating cells were stimulated with phorbol ester and ionomycin and IFN-γ was detected by intracellular staining (**Table 1**). No difference in the percentage of IFN-γ–producing T cells was found in CD27$^{+/+}$ and CD27$^{-/-}$ mice. This indicated that a similar proportion of CD4⁺ and CD8⁺ T cells present in the lungs of WT and CD27-deficient mice had completed the differentiation program that allows IFN-γ production.

Discussion

Our results show that CD27 is not essential for generation and maintenance of T and B lymphocyte populations. Previously, we hypothesized that CD27 acts as a costimulus for pre-TCR function[35], based on the finding that *in vivo*–administered mAb to CD27 inhibited generation of the CD4⁺CD8⁺ thymic compartment. However, T cell development was normal in CD27-deficient mice so we inferred that CD27-CD70 interaction was not required to support pre-TCR function. In our earlier experiments[35], mAb to CD27 presumably induced a CD27 function that counteracted the pre-TCR-induced response.

The CFSE assay showed that lack of CD27 expression in CD27-deficient cells or stimulation of CD27 with specific mAb in WT cells does not affect cell cycle activity of activated peripheral T cells. Yet both interventions affect [³H]thymidine incorporation[20] (**Fig. 2a**). This can be explained by the notion that incorporation of [³H]thymidine is not only determined by cell cycle activity, but also by the number of cells participating in the response. The latter is determined by entry into cell cycle and by cell survival. As we have clearly demonstrated that CD27 does not affect the cell division process, reduced [³H]thymidine incorporation in CD27$^{-/-}$ T cells is most likely due to decreased T cell survival.

In contrast to CD28, CD27 employs Traf molecules to induce downstream signals, in particular Traf-2 and Traf-5[36,37]. Whereas in Traf-5$^{-/-}$ mice, lymphocyte homeostasis does not seem to be affected[38], Traf-2$^{-/-}$ mice are severely lymphopenic, implicating this molecule in T and B cell surivival[7]. Analysis of the virus-specific response of CD4⁺ T cells, isolated from the spleens of immunized mice confirmed that CD27 is essential for adequate expansion of antigen-activated T cells.

Studies suggest that CD27 may contribute to the generation of cytolytic effector cells[13,30]. However, in these experiments, stimulatory effects of CD27 on T cell expansion may have obscured the results as the actual amount of antigen-specific T cells entered into the cytotoxicity assay was not determined. In our experiments equal amounts of peptide-specific T cells were used, which allowed the conclusion that CD27 does not stimulate the differentiation program

per se. Similarly, we found that CD27-deficient T cells of both $CD8^+$ and $CD4^+$ phenotype can adequately synthesize IFN-γ.

Primary influenza virus–specific responses of both $CD4^+$ and $CD8^+$ T cells were affected in $CD27^{-/-}$ mice. The absolute numbers of T cells infiltrating the lung, the number of tetramer-positive $CD8^+$ T cells in lung and spleen and the anti-viral response of splenic $CD4^+$ T cells *in vitro* provided evidence for this conclusion. Among Traf-linked TNF receptor family members, CD40[39–41], 4-1BB[42,43] and OX-40[44,45] are implicated in T cell priming. CD40 plays a role on DCs, interacting with CD40L on both $CD4^+$ and $CD8^+$ T cells[39–41]. Apart from skewing cytokine production by DC, it serves to up-regulate the ligands for CD28, such that costimulation of the T cell can take place[4]. Yet on infection with lymphocytic choriomeningitis virus (LCMV), which causes a systemic infection, the cytotoxic and helper T cell responses are not critically dependent on either CD28[46] or CD40L[47,48]. A role of the different costimulatory molecules may be revealed depending on the model system chosen. For instance, after localized infection with influenza virus, primary $CD8^+$ T cell responses are reduced in the absence of CD28 function[49,50].

The molecules 4-1BB and OX-40 are found on activated T cells and can interact with ligand on DCs[24,25]. Mice that are $4\text{-}1BBL^{-/-}$ can generate LCMV-specific CTLs and clear the virus[42,43], but virus-specific $CD8^+$ T cell numbers were reduced[43]. In $OX\text{-}40^{-/-}$ mice, the number of lung-infiltrating $CD4^+$, but not $CD8^+$ T cells was reduced after influenza virus infection. Splenic $CD4^+$ T cells showed deficient expansion and cytokine production in response to both influenza virus and LCMV. However, primary CTL responses to these viruses were normal[44]. Also, $CD4^+$ T cells taken from OX-40L–deficient mice immunized with protein antigens showed impaired responsiveness upon rechallenge *in vitro*. In this assay, $OX\text{-}40L^{-/-}$ antigen presenting cells were nonfunctional, pinpointing the defect to T cell–DC communication[45]. Other assays in $OX\text{-}40L^{-/-}$ mice revealed a defect in $CD8^+$ T cell–dependent contact hypersensitivity[51]. Apparently, the OX-40–OX-40L pair is involved in certain, but not all, T cell–DC interactions and is not selective for $CD4^+$ or $CD8^+$ T cells. CD70 has been detected on activated mouse DCS[27]. Therefore, the CD27-CD70 pair may contribute to T cell priming at the DC–T cell interphase, like 4-1BB and OX-40. In addition, both CD70 and OX-40L are expressed on activated T cells and can support T cell expansion during T–T cell interaction.

The most dramatic defect in $CD27^{-/-}$ mice concerned T cell memory, in particular of $CD8^+$ T cells. Memory is dependent on the amount of surviving T cells after primary TCR-mediated activation and presumably on escape from activation-induced cell death. Traf-linked TNF receptor family members, by virtue of their anti-apoptotic effects, would be prime candidates to shape T cell memory. However, thus far, only CD40L has been implicated in T cell memory using gene-targeted mice[52]. OX-40 was shown to inhibit peripheral deletion and enhance long-term T cell survival by *in vivo* antibody injection studies[53]. CD27 may exert its effects by promoting T cell survival and/or expansion of memory T cells. We cannot exclude a role for CD27 in lymphocyte trafficking. Recently, its close relative OX-40 was connected to cell migration. OX-40 was found to up-regulate expression of the CXCR5 chemokine receptor and allow T cell migration into B cell areas of the peripheral lymphoid organs[54].

Although the $CD27^{-/-}$ mice had reduced T cell immunity, in particular the $CD8^+$ T cells, they did not become overtly more ill and recovered from influenza virus infection within the same time period as WT mice. In part, this may be explained by the finding that $CD27^{-/-}$ mice have a normal B cell response to influenza virus. Serum titers of virus-specific immunoglobulins of all isotypes are in the same order of magnitude as in WT mice (data not shown). Also, immunoglobulin levels remain high in both $CD27^{-/-}$ mice and WT mice for more than 3 months after primary infection and therefore are expected to contribute to virus elimination after secondary challenge.

This study underlines the importance of Traf-linked TNF receptor family members in induction and maintenance of the specific immune response. These molecules provide a lead for the development of alternative, subtle immunological intervention for conditions of excessive or deficient immune responsiveness, such as autoimmunity and cancer.

Methods

Mice. All mice were bred in the facility of The Netherlands Cancer Institute under specific pathogen-free conditions and used for experiments at 6 to 8 weeks of age. All animal experiments were carried out according to institutional and national guidelines and approved by the Experimental Animal Committee of The Netherlands Cancer Institute (DEK).

Generation of CD27-deficient mice. Clone λ5 was isolated from a genomic 129/OLA DNA library in phage λFIX (Stratagene, La Jolla, CA) by probing with a 5′ 600-bp *Eco*RI-*Pst*I fragment of the murine CD27 cDNA[12]. *Xba*I and *Xba*I-*Not*I fragments of λ5 were subcloned and characterized by Southern blotting with CD27 cDNA and by nucleotide sequencing. To make the targeting construct, a λ5 fragment containing the entire coding region, from the *Bgl*II site just upstream of the ATG codon to the *Bam*HI site, downstream of the stop codon, was replaced by the hygromycin resistance gene under the control of the GK promoter. Subclone IB10, derived from the 129/OLA ES cell clone E14 was electroporated with a *Not*I-*Sal*I fragment of the targeting construct. Genomic DNA of ES cell clones resistant to 150 μg/ml hygromycin B (Calbiochem, La Jolla, CA) was diagnosed by Southern blotting, as described in the Results.

Genotypic analysis of mice. The mutated *Tnfrsf7* allele was detected by PCR on tail DNA of the hygromycin B–resistance gene, using nucleotides 256–274 as sense and 914–933 as antisense primers. The *Tnfrsf7* WT allele was detected using nucleotides 869–889 of the cDNA as sense and 1236–1255 as antisense primers[12].

Flow cytometry. Peripheral blood was collected and cells were spun down. Erythrocytes were then lysed in 0.14 M NH_4Cl, 0.017 M Tris-HCl at pH 7.2 on ice for 10 min. Peripheral blood cells and cells from bone marrow, thymus, spleen and lymph nodes were preincubated with an Fc Block (mAb to CD16–CD32, 2.4G2, PharMingen, San Diego, CA) and washed in FACS buffer (PBS, 0.5% bovine serum albumin, 0.01% sodium azide). Next, they were incubated with specific antibody directly conjugated to fluorsecein isothiocyante (FITC), phycoerythrin (PE) or biotin as indicated, washed, incubated with Tricolor-streptavidin or PE-streptavidin (Caltag, San Diego, CA) when applicable, washed and analyzed using a FACScan and LYSYS II software (Becton Dickinson, Mountain View, CA). Immunofluorescence mAbs were from PharMingen or donated by investigators. The mAbs we used were anti-CD3ε, 500A2; anti-CD4, RM4-5; anti-CD8α, 53-6.7; anti-CD27, LG.3A10[20], anti-CD28, 37.51; anti-CD44, IM7; anti-CD45R (B220), RA3.6B2; anti-CD69, H1.2F3; anti-IgM, R6-60.2; anti-IFN-γ, XMG1.2. PE-labeled tetramers of the murine class I molecule H2-D^b, β_2-microglobulin and the influenza nucleoprotein peptide ASNENMDAM, NP(366-374), were prepared as described[32]. Cells were incubated with MHC class I tetramers together with FITC–anti-CD8α. Cells were washed in FACS buffer and analyzed using a FACSCalibur and CELLQuest software (Becton Dickinson, Mountain View, CA).

Preparation of purified T cells. Lymph nodes and spleens were dissociated in Iscove's Modified Dulbecco's medium supplemented with 8% fetal calf serum (FCS), penicillin and streptomycin. Erythrocytes were lysed in 0.14 M NH_4Cl, 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 mAb M5/114 to MHC class II. In case of $CD4^+$ T cell purification, 2 μg of mAb to CD8α per 10^6 cells was added. This was followed by a 30-min incubation on ice with 100 μl of goat anti-mouse immunoglobulin-coated magnetic beads and 20 μl of goat anti-rat immunoglobulin-coated magnetic beads per 10^7 cells (Advanced Magnetics Inc., Cambridge, MA). Purity of the resulting cell population was checked by immunofluorescence analysis with mAbs to CD3ε and B220. Only preparations that contained more than 98% T cells or $CD4^+$ T cell preparations that were more than 90% pure were used.

***In vitro* T cell stimulation**. Purified T cells from backcrossed mice (10^5 per well) were plated in IMDM with 8% FCS in 96-well round-bottom plates, coated with mAb 145-

2C11 to CD3ε at the indicated concentrations, in the presence or absence of coated mAb 37.51 to CD28 (10 μg/ml) or IL-2 (100 U/ml). Cells were cultured for 3 days. During the last 8 h of culture, 0.4 μCi [^{3}H]thymidine (Amersham International, Amersham, UK) was present per well. Proliferation of virus-specific CD4$^+$ T cells was determined using 2×10^5 purified spleen CD4$^+$ T cells per well, taken from mice at day 14 after primary infection or at day 11 after secondary infection. Sucrose gradient–purified influenza virus was inactivated with 2% paraformaldehyde in PBS for 20 min at room temperature followed by addition of glycin to 0.2 M and overnight dialysis in PBS. Virus was added to the culture at indicated concentrations. Irradiated (25 Gy) C57BL/6 spleen cells were used as antigen presenting cells at 4×10^5 cells per well. Cells were cultured for 4 days; for the last 8 h in the presence of [^{3}H]thymidine. Cells were collected with a Titertek automatic harvester (ICN, Costa Mesa, CA) and incorporation of radioactivity was measured in a β-plate counter (Pharmacia, Uppsala, Sweden). For cell cycle analysis, purified T cells were labeled with 5 μM of CFSE (Molecular Probes) before stimulation. Cells were stimulated with coated mAb to CD3ε with or without coated mAb to CD28 (both at 10 μg/ml), or soluble mAb to CD27 (0.1 μg/ml). Gated live cells were analyzed by flow cytometry after 3 days of culture.

Cytotoxicity assay. Cytolytic activity of virus-specific CD8$^+$ T cells derived from spleens from infected mice was determined in a 5-h ^{51}Cr-release assay. Spleens from mice at day 14 after primary infection or day 11 after secondary infection were dissociated into single cell suspensions and restimulated for 7 days in the presence of 0.5 ng/ml of NP-derived peptide ASNENMDAM and 20 U/ml of recombinant human IL-2. EL-4 target cells were preincubated with peptide for 1 h at 37 ºC. Percent specific lysis was calculated from the equation:

$$\frac{(\text{experimental } ^{51}\text{Cr-release} - \text{spontaneous } ^{51}\text{Cr-release})}{(\text{maximum } ^{51}\text{Cr-release} - \text{spontaneous } ^{51}\text{Cr-release})} \times 100 = \% \text{ specific lysis.}$$

Intracellular cytokine staining. Lung-infiltrating cells were stimulated for 5 h with 5 ng/ml of phorbol 12-myristate 13-acetate and 1 μM of ionomycin in the presence of 10 μg/ml of brefeldin A (Sigma, St. Louis, MO). Cells were collected and washed once with FACS buffer. After incubation with Fc Block and staining with PE-conjugated mAb to CD4 and FITC-conjugated mAb to CD8, cells were fixed with 4% paraformaldehyde in PBS for 5 min. They were washed once with FACS buffer, containing 0.1% saponin (Sigma) for permeabilization, and incubated in the same buffer with allophycocyanin-conjugated mAb to IFN-γ for 30 min on ice. Cells were washed three times, resuspended in FACS buffer and analyzed by flow cytometry.

Virus infection. Influenza virus strain A/NT/60/68 was grown, purified and tested for hemagglutinin activity and infectious titers in the Department of Virology, Erasmus University Rotterdam, The Netherlands. Mice used in these experiments were backcrossed at least seven times to the C57BL/6 background and CD27-deficient and WT littermates were compared in all experiments. Mice were anesthetized and infected intranasally with 50 μl of PBS with or without 25 or 200 HAU of virus for primary and secondary infections, respectively. Mice were killed at indicated time points after infection, and lungs and spleen were removed. They were minced in single chamber mesh filters and cells were counted and analyzed by flow cytometry.

Statistical analysis. All statistical analyses were based on ANOVA. A one-quarter power transformation was applied to achieve normally distributed data. *P* values were calculated from type II sum of squares. Bonferroni correction (multiplication of the *P* values by 4) was used to correct for the probability of false positive findings due to use of four response measures.

Acknowledgements

We thank E. Tanger, M. Timpico, L. Tolkamp, T. Schrauwers, H. Starrevelt and other staff of the Animal Facility of the Netherlands Cancer Institute for biotechnical assistance and maintenance of the mice; A. A. M. Hart for expert statistical analysis; G. Rimmelzwaan for virus preparations and advice; E. Noteboom and A. Pfauth for assistance with flow cytometry; P. Krimpenfort, J. Haanen, G. Dingjan, R. Hendriks and J. Kirberg for experimental advise and assistance; and A. M. Kruisbeek for reading the manuscript. Supported by The Netherlands Organization for Scientific Research (NWO).

Received 18 August 2000; accepted 6 October 2000.

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