Contributions of CD27 and relatives to the specific immune response
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Chapter 3

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 receptor. The tumor necrosis factor (TNF) receptor family contains death receptors, as well as receptors that bind TNF receptor-associated factors (Traf). 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. Other prominent members are RANK and CD40, which additionally modulate the response of dendritic cells (DCs). 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. Traf signal to NF-xB and Jun kinase, but the array of genes they target is undefined. It is proposed that Traf counteract apoptosis via the inhibitor of apoptosis proteins (IAPs) and/or NF-xB.

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. CD27 is found on NK, T and B cell populations. CD27 ligand expression is up-regulated upon TCR stimulation on both T and B cells and thus reflects recent antigenic priming. CD70 is also found on thymic DCs in humans and on lymph node DCs from infected mice, but not on any other cell types. CD70 makes essential contributions to mature 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 definitive localization of CD27 in the immune system where it plays 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. Other prominent members are RANK and CD40, which additionally modulate the response of dendritic cells (DCs). 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. Traf signal to NF-xB and Jun kinase, but the array of genes they target is undefined. It is proposed that Traf counteract apoptosis via the inhibitor of apoptosis proteins (IAPs) and/or NF-xB.

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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 Scal 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 Tnfrsf? 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. It also confirmed the CD27-deficient phenotype (Fig. 1c). CD27- mice proved viable, fertile and remained healthy over prolonged periods of time (>6 months).

**Flow cytometric analysis of spleen cells from CD27- mice and CD27+ mice. Cells were double stained as indicated.**

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 [3H]thymidine incorporation is enhanced by monoclonal antibody (mAb) to CD27 or recombinant ligand. Conversely, mAbs to CD70 inhibit this response, indicating that CD27-CD70 interaction supports TCR-induced T cell expansion. Purified, naïve peripheral T cells from CD27- mice showed significantly decreased TCR-induced [3H]thymidine incorporation as compared to WT T cells, but did not rescue the defect in CD27- cells (Fig. 2a). Thus, reduced [3H]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 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
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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. lc) and costimulation with mAb to CD27 enhances [3H]thymidine incorporation three- to fourfold-3 T cells express CD27 (Fig. lc) and costimulation with mAb to CD27 enhanced [3H]thymidine incorporation three- to fourfold-3 T cells express CD27 (Fig. lc) and costimulation with mAb to CD27 enhances [3H]thymidine incorporation three- to fourfold. We conclude that, unlike CD28, CD27 does not influence the cell division process. Its effect on [3H]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-Dd tetramers loaded with the immunodominant influenza NP(366–374) peptide allow visualization and quantification of virus-specific CD8+ T cells. 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 20-30×10^6 virus-specific T cells in the spleen. In the 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 CD8+ 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 (60×10^6 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
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
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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 ratio 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, based on the finding that in vivo-administered mAb to CD27 inhibited generation of the CD4+CD68+ 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, 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. 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, mAb to CD27 presumably induced a CD27 function that counteracted the pre-TCR-induced response.

In contrast to CD28, CD27 employs Traf molecules to induce downstream signals, in particular Traf-2 and Traf-5. Whereas in Traf-5- mice, lymphocyte homeostasis does not seem to be affected, Traf-2- mice are severely lymphopenic, implicating this molecule in T and B cell survival. 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. 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 entering 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.
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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, CD40L+1, 4-1BB+24 and OX-40+9, 12 are implicated in T cell priming. CD40 plays a role on DCs, interacting with CD40L on both CD4+ and CD8+ T cells. 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. Yet on infection with lymphocytotoxic or encephalitogenic virus, CD27 may exert its effects by promoting T cell survival during latency 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. Among Traf-linked TNF receptor family members, CD40 plays a role in CD8+ T cell survival and the anti-viral response of splenic CD4+ T cells.

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.

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 peri-

od 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 3.5 was isolated from a genomic DNA library in phage λgt11 (Stratagene, La Jolla, CA) by probing with a 5’ 600-bp EcoRI–BglII fragment of the murine CD27 cDNA+13, 16 and Xhol–NotI fragments of 3.5 were subcloned and characterized by Southern blotting with CD27 cDNA and by nucleotide sequencing. To make the targeting construct, a 5.5 fragment containing the entire coding region, from the BglII site just upstream of the ATG codon to the BamHI site, downstream of the stop codon, was replaced by the hygromycin resistance gene under the control of the CMV promoter. Subclone B10, derived from the 129/OLA x A cell clone El4 was electroporated with a Not-Sall fragment of the targeting construct. Genomic DNA of ES cells resistant to 150 μg/ml hygromycin B (Calbiochem, La Jolla, CA) was diagnosed by Southern blotting, as described in the Results.

Flow cytometry. Peripheral blood was collected and cells were spun down. Erythrocytes were then lysed in 0.14 M NH4Cl, 0.107 M Tris·HCl at pH 7.2 in ice for 10 min. Peripheral blood cells and cells from bone marrow, thymus, spleen and lymph nodes were suspended 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 fluorescein isothiocyanate (FITC), phycoerythrin (PE) or biotin as indicated, washed with Tricolor-strepavidin or PE-strepavidin (Caltag, San Diego, CA) when applicable, washed and analyzed using a FACScan and LYSYS II software (Becton Dickinson, Mountain View, CA). Immunofluorescence was confirmed and approved by the Experimental Animal Committee of The Netherlands Cancer Institute (DEK).

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 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 mAb 53-6.7 (anti-CD8, IgM, R6-60.2, anti-IFN-γ, BMC-1, PE-labeled tetramers of the murine class I molecule H-2D^P, β2-microglobulin and the influenza nucleoprotein peptide ASN697DNAH, NP606-374), were prepared as described+3. Cells were incubated with MHC class I tetramers together with FITC–anti-CD69. Cells were washed in FACS buffer and analyzed using a FACS Calibur and CELLQuest software (Becton Dickinson, Mountain View, CA).

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2C11 to CD3E at the indicated concentrations, in the presence or absence of coated C57BL/6 spleen cells were used as antigen presenting cells at 4x10^6 cells per well. PBS. Virus was added to the culture at indicated concentrations. Irradiated (25 Gy) spleens from infected mice was determined in a 5-h Cytotoxicity assay. Cytolytic activity of virus-specific CD8* T cells derived from the determinant with allophycocyanin-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 PE-labeled mAb to IFN-γ for 30 min on ice. Cells were washed three times, resuspended in FACS buffer and analyzed by flow cytometry. Intranasally with 50 μl of PBS with or without 25 or 200 HAU of virus for primary and CD40 molecules and down regulation by TGF-β. Exp. Med. 188, 1679-1685 (1998).


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