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THE TWO-SIGNAL MODEL OF T-CELL ACTIVATION AFTER 30 YEARS

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The two-signal model of T-cell activation is still valid after 30 years. The recent understanding of the first signal intricacy and its tight relationship with the second signal have thrown decisive light on T-cell activation processes and the complex molecular events that occur on the surface and within the T cell. Furthermore, the recognition of numerous accessory pathways that, in addition to the CD28 and CD40 pathways, operate to manage antigen-presenting cell T cell cooperation in view of lymphocyte activation, disclose the exquisite and numerous regulatory events that compose the second signal.

Few biological models have remained in use for more than 30 years. No doubt the two-signal model of lymphocyte activation is among these happy few. Derived from a two-signal model originally proposed by Bretscher and Cohn (*1*) in 1970 in an attempt to account for self-tolerance in the periphery, the model in its present form assumes that T cells require two signals to be activated by antigen-presenting cells (APC). The first signal is delivered via T cell receptors (TCR) upon antigen presentation. The second signal has two important features; (i) it has no cognition of the antigen, resulting from receptor counter-receptor interactions not related to antigen specificity, and (ii) it can be produced by a number of distinct molecular interactions that may occur between an APC and a T cell.

THE KINETIC ASPECTS OF T-CELL ACTIVATION: THE FIRST SIGNAL IS TIME REQUIRING

Although the molecular interactions that support the first signal at the APC T cell interface are well identified (*2*), the first signal has many intimate relationships with the second signal. The binding of TCR with a peptide-loaded class II molecule is very brief (*3*). Therefore the question to be addressed is how many of these interactions are required to deliver a consistent activation signal? Assuming that TCR internalization, which is a rapid and sensitive event, occurs whenever a given TCR gives rise to a "hit," Lanzavecchia and Sallusto (*3*) have counted that the engagement of 8000 TCR were required to activate a virgin T cell; should a coactivation signal be delivered via CD28, then the number of engagements required is lowered to 1500 TCR hits. Thus, given that the virgin T cell carries 10,000–40,000 TCR, a considerable proportion of the available TCR must be engaged.

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This also means that the second signal not only delivers a qualitatively distinct activation signal (see below) but also acts synergistically with the first signal. This appears clearly when attention is focused on the intracellular second messengers raised via these activation molecules.

Overall, the interactions that must occur between APC and virgin $CD4^+$ T cells require 20 hr to give full signalization, whereas only 20 min are required for memory T cells (*3*). Because the life span of activated dendritic cells (DC) in the T-cell zone of a lymph node is 48 hr, this is the first limitation in the activation process which remains dependent on the influx of DC. Interestingly, recent data (*4*) have shown that virgin $CD8^+$ T cells are much more ready to go than CD4 cells, provided that sufficient activated APC provide the required signals to CD8.

REORGANIZATION ABOVE AND BELOW THE CELL SURFACE: THE T CELL "SIGNALOSOME" SCAFFOLD AND THE T-CELL SYNAPSE

For many years, it has been clear that a long physical coupling must occur between APC and T cells, thus leading to sustained adhesion pathways. Two such pathways can maintain significant avidity between the two cellular partners: the CD2 pathway and the lymphocyte function-associated antigen (LFA)-1/intercellular adhesion molecule (ICAM) pathway. While the issue of the CD2 pathway remains controversial and inadequately understood, the role of the $\alpha L\beta 2$ integrin (LFA-1) pathway seems to be more straightforward. As the cells become polarized, adhesion events are accompanied by spectacular changes both within the cell and on the cell surface.

A likely scenario of early events could be this: upon interaction between a TCR and a peptide-loaded Cl II, the fastest consequence seems to be the inclusion of TCR within membrane glycolipid-rich microdomains (*5*). This facilitates approximation of TCR with signalization molecules, in particular Src kinases (Lck and Fyn), together with cytoskeletal elements, both known to be abundant within these microdomains. At present, it is still unclear whether the activation signal requires TCR cross-linking; nevertheless, "activated" TCR accumulate at the contact area between T cells and APC. The first phosphorylation of tyrosine residues occurs on the TCR-CD3 ζ chain, most likely by Fyn and Lck, allowing binding of the ZAP kinase on the phosphorylated ITAM motifs of CD3ζ. In turn, this allows binding of the src kinases to ZAP70, initiating the signaling scaffold. Because Lck is bound to the intracytoplasmic segment of CD4 or CD8, the appearance of Lck binding sites on the CD3ζ/Zap70 scaffold facilitates the approximation of CD4, or CD8, with the "right" TCR, namely those which have been effectively hit by a peptide-loaded Cl II or Cl I. The subsequent binding of CD4 or 8 to constant regions of HLA Cl II or Cl I molecules "locks"

the contact with TCR. Actin fasciculation is initiated, almost as quickly, and this ultimately propagates to whole cells, while the most profound cytoskeletal reorganizations will remain focused at the CD3ζ contact. Thus, within the first minute, an orderly accumulation of second messengers and cytoskeletal elements occurs on the intracytoplasmic side.

One of the first consequences of an activation signal "getting through" the TCR signalosome is activation of local LFA-1 integrins. On resting cells, these integrins do not display a significant affinity for their ICAM ligands; they have limited contact with the cytoskeleton. Signalization via the TCR produces an "outside-in" effect. LFA-1 integrins unmask a high-affinity binding site for ICAM and reinforce their linkage to the cytoskeleton with strong actin fasciculation. They group together, forming focal contact areas, thereby increasing both their affinity and T-cell avidity for APC. At this stage, the binding of the two cellular partners is firmly ensured. In turn, LFA-1 delivers a strong coactivation signal within the T cell, which can be considered as a first occurrence of a "second signal." In the area of cell contact, signaling and adhesion molecules accumulate, forming what is now called the T-cell synapse. Membrane-bound molecules are not evenly distributed in this area. Dustin and Cooper (*6*) have observed that within 1 min, LFA-1 integrins are located within an inner circle where the tightest contacts occur, whereas the TCR are located in a peripheral circle. After 5 min, while T cells have flattened, the distribution changes dramatically and integrins are accumulated in the peripheral ring where strong actin cables are anchored. The inner circle now contains TCR, CD4, or CD8 molecules, as well as the signaling molecules responsible for the second signal.

THE SECOND SIGNAL PROTOTYPE: THE SELF-AMPLIFIED AND SELF-INHIBITED CD28 STARTER PATHWAY

CD28 is the prototype molecule that delivers a second signal (*7*). It is required to activate virgin T cells, particularly in view of T–B cooperation: CD28-deficient mice have impaired T cell-dependent B-cell responses but no defect in the generation of CD8 cytotoxic cells. When crossed with susceptible mice, they usually show a reduced severity of autoimmune diseases. Memory CD4 cells do not require CD28 for reactivation. This observation led to the schematic view that no cosignal is required to activate memory cells. An interesting aspect of the CD28 pathway is that it is self-amplified and self-limited. On resting naive T cells, CD28 is constitutively expressed, whereas on APC, the constitutive counter-receptor CD86 (B7-2) is also present, although at a rather low density. Both molecules can thus interact as soon as the synapse is formed, when both membranes are in sufficiently close contact. This cosignal enables the full process of activation to be initiated; it is rapidly amplified because CD28 and CD86 increase their expression in the first hours of the activation process, and a synergistic effect occurs with the developing CD40L (CD154)–CD40 pathway (see below). However, after 2 days, two new surface molecules appear: CTLA-4 (CD152) on T cells and CD80 (B7-1) on APC. The affinity between these two molecules is extremely high, although CD80 shows good interaction with CD28. Thus in experimental systems in which CD152/CTLA-4 is lacking or blocked, CD80/B7-1 may contribute to stimulating T cells. Nevertheless, after 2 days in normal conditions, the interactions within the CD28 pathway are fully governed by CTLA-4/CD152–CD80 interactions that deliver a strong inhibitory signal within the T cell. T-cell activation then ceases. CTLA-4/CD152-deficient mice have aggravated polyclonal proliferative responses with a fatal issue at a young age; T cells displaying an activated and memory phenotype accumulate in the lymphoid organs (*8*).

The CD28 pathway is a key pathway to initiating primary immune responses. Although CD28 seems to facilitate a T helper (Th)2 response, it is still unclear whether it can decide on a Th2 versus Th1 polarization. Moreover, CD28 costimulation is required for important effects that may seem to be contradictory in different experimental systems. For instance, B7-mediated signaling is mandatory for inducing the chemokine receptors required for the appropriate T cells to migrate in the right place. Attenuation of the immune response in vivo may be partly due to this effect. On the other hand, the CD28 pathway also seems necessary for the development of the newly described $CD25^+$ CD4 cells and could explain the paradoxical aggravation of some autoimmune diseases in CD28-deficient mice. The numerous attempts to manipulate the immune response by acting on the pathway have led to promising but sometimes unexpected or contradictory results.

THE CD28 PATHWAY IS ONLY PART OF A COMPLEX NETWORK INCLUDING MANY STRUCTURALLY RELATED PATHWAYS

It now seems that the CD28 pathway is only part of a large network including several other pathways that act together to control T- or B-cell activation. From a structural standpoint, these pathways belong to two distinct families: the CD28–B7 family including Ig-like molecules, and the tumor necrosis factor (TNF)–TNR receptor (TNF-R) family including nerve growth factors (NGF) and NGF receptor-like molecules. These two families will now be successively reviewed, although it is important to bear in mind that their recent discovery has left many uncertainties or may well be followed in the near future by unexpected developments. It must also be emphasized that it is semantically incorrect to address the question of their functional involvement as isolated pathways—which we shall do now for clarity—because they do not work alone but as integrated members of a network of pathways that act in concert.

THE FAMILY OF CD28–B7 MOLECULES; THE CD28-RELATED PATHWAYS

Within the CD28–B7 family, T-cell surface molecules, on the one hand, and APC surface molecules on the other hand, are all similar (Figs. 1 and 2). The CD28-like molecules are made of a single V-like Ig domain extended by a stalk from the T-cell surface. They form homodimers and, apart from PD-1 (see below), have their genes coded in the 2q33 region. On the APC surface, B7-like molecules are made of a distal V-like and a proximal C-like Ig domain and most probably also form homodimers. They are coded in the 2q23 region. The crystal structure of CD28, CD86, and CD152/CTLA-4 has recently been established (*9*). This shows that, while the Ig domain(s) of CD28 and CD86 extend perpendicularly to the cell surface, the CTLA-4 domain is rotated parallel to the cell surface. Therefore it is likely that, given the dimerizations that occur between the counter-receptors, CTLA-4/CD152 and B7-2/CD86 can form an extended structure that covers the synapse. This may contribute to further prevent-

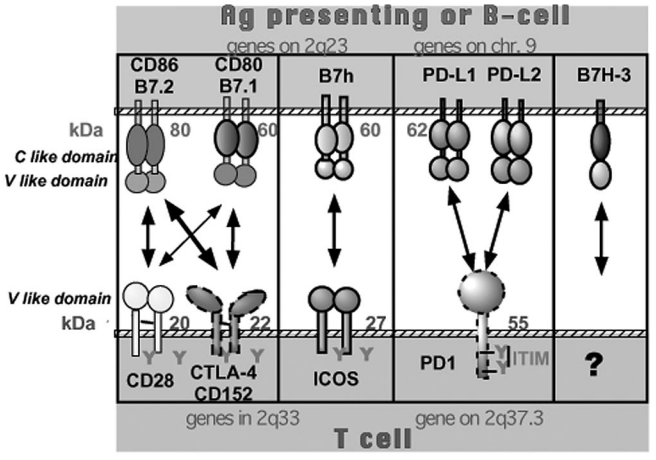

FIGURE 1. The molecules and pathways of the CD28–B7 family.

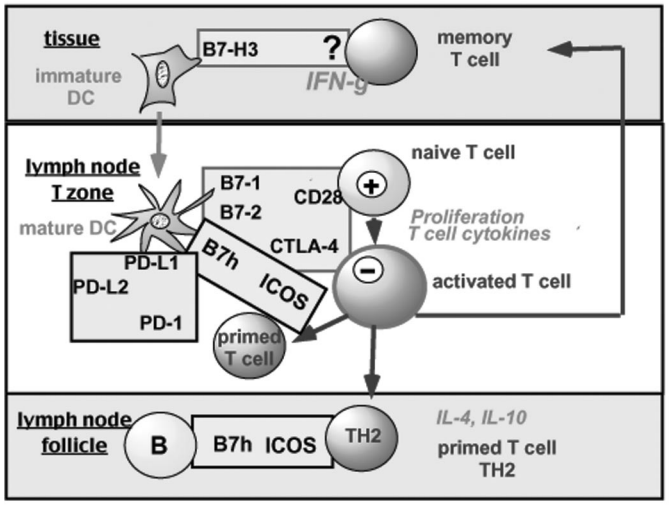

FIGURE 2. Diagram of functional involvement of the CD28–B7 family pathways.

ing T-cell activation for mechanical reasons, in addition to the active negative signal transmitted via CD152.

The Inducible Costimulator (ICOS)–B7h pathway

This pathway appears after 2–4 days of primary T-cell activation (*10*). The molecules defining this pathway were cloned upon completion of the genomic characterization of this region. B7h molecules (also called B7RP-1, B7H2, LICOS, and GL50) are now recognized as a ligand for ICOS. B7h were cloned by subtractive hybridization as homologous to CD86 and CD80 (approximately 20% homology). They are not expressed on resting monocytes but induced by adhesion or interferon (IFN)-γ. B7h persist on DC all along their activation. While B7h knockout (KO) mice have not as yet been described, several groups have described ICOS KO mice. They display severe impairment of primary T helper cell responses and a reduced switch of Ig. These defects can be partly corrected by stimulation of the CD40 pathway, indicating a synergy between both pathways. It is of note that ICOS preferentially induce the production of interleukin (IL)-2 and IL-4, but neither IFN-γ nor IL-10, and therefore seem to favor a Th2 response. Beyond the primary immune response, ICOS can quickly re-expand memory cells, even though CD28 and CD40 are deficient. Finally, in addition to their role in APC T cell cooperation, ICOS may play a direct role in B-cell activation in which they are present in resting and germinal center (GC) cells because ICOS KO mice have no GC.

The PD-1–PDL-1 and 2 pathway

PD-1 resembles CD28, CD152/CTLA-4, and ICOS. However its gene is located in 2q37.3 and its intracytoplasmic segment includes an ITIM motif shared with inhibitory molecules. Indeed mice with an invalidated Rd.1 gene for PD-1 are susceptible to autoimmune diseases and, interestingly, do not display the same disorders depending on their genetic background (*11*). C57Bl6 PD-1 −/− mice suffer from lupus-like syndrome and arthritis, whereas BALB/C PD-1 −/− mice suffer from dilated cardiomyopathy, with IgG deposit and autoantibodies. When crossed with mice expressing a transgenic autoreactive TCR, PD-1 deficiency produces a graft-versus host syndrome with infiltration of inflammatory cells and peripheral T cells with memory-activated rather than virgin type. PD-1 is present on activated T, B, and myeloid cells. It has two recognized ligands termed PDL-1 and PDL-2. Both consist of a terminal V-like Ig domain and a proximal C type Ig domain like B7 molecules, yet their genes are located on chromosome 9. The important point about PDL-1 is that its expression is induced in DC upon their activation but is constitutive on peripheral cells of many organs. It is therefore clear that the PD-1 pathway is an inhibitory pathway that is likely to act synergistically with CD152/CTLA-4 to help switch off T-cell responses against nonself-antigens by acting on DC T cell cooperation. In addition, it would be shaped to help maintain peripheral tolerance against undue activation of self-reacting clones by acting in the periphery. By preventing further APC activation that occurs via the CD40L–CD40 pathway, it prevents "hyper" activation of APC and subsequent CD8 cell activation as seen above.

Further CD28-Like Pathways?

One can safely suppose that other unknown pathways of the family are activated; an additional B7-like molecule, termed B7H3 or B7RP-2, has recently been discovered (*12*). It is closely related to B7h, for which no CD28-like counter-receptor has yet been found. It is highly expressed on immature DC but, in contrast to B7h, is lacking on B cells. It is a potent stimulator of CD4 cells, particularly for inducing IFN-γ production and enhancing the cytotoxic functions of CD8 cells. However, whether it belongs to a "short circuit" that would act directly in the periphery when a second antigenic aggression occurs remains unknown.

THE OTHER LARGE FAMILY: THE TNF/TNF-R-LIKE FAMILY, OR WHETHER A T CELL WILL DIE OR LIVE AND BE ACTIVATED

The molecular pathways belonging to this family all share similar peculiar structural features, organization, and chromosomal locations (Fig. 3). The TN-like ligands spontaneously form trimers and can be expressed, in various proportions, as a cell surface receptor or as a soluble factor after detachment by metalloproteases; their counter-receptors resemble one another because they are type II transmembrane molecules that include various numbers of cysteine-rich domains. One view was that the counter-receptors form trimers upon binding of the TNF-like ligand, either

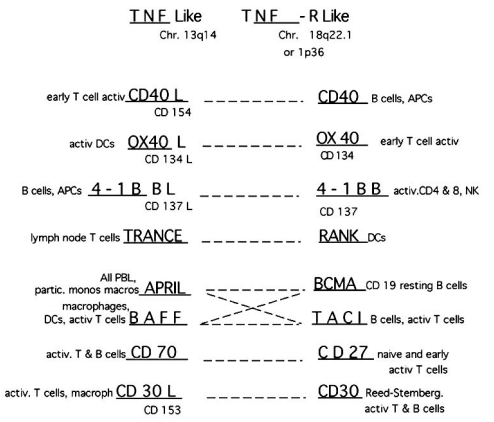

FIGURE 3. The activator pathways of the TNF–TNFR-like family.

as a cell surface molecule or as a soluble factor. More recent data indicate that they also spontaneously form trimers due to a peculiar motif located in the terminal cysteine-rich domain. The counter-receptors, including cysteine-rich domains, can transduce, within the carrying cells, powerful signals that are decisive for the fate of the cell. Either they activate nuclear factor-κB (NF-κB) and/or Rel and transduce within the cell mitogenic and surviving signals, or they activate caspases, via a linking molecular complex (termed DISC) and transduce a strong pro-apoptotic signal causing imminent cell death. Of note is the fact that whenever a death signal is transduced, it is not always a result of the presence of the so-called death domain within the cytoplasmic segment of the TNF-R-like molecule. Note also that the fate is univocal and simply dictated by the appearance or not of the ligand-counter-receptor on a couple of interacting cells. Thus, in the case of the Fas ligand (TNF-like)–Fas (TNF-R-like) pathway, the presence of both molecules is sufficient to lead the Fas-carrying cell to death. Thus the Fas ligand–Fas system is an ultimate safeguard in T-cell activation because, after several days of activation or reactivation, the vast majority of activated T cells display both Fas and Fas-L leading to propriocidal or even suicidal killing. Mice deficient in either of the molecules accumulate activated or memory cells in a type of polyclonal lymphoid proliferation.

THE DECISION TO LIVE: THE CD40L–CD40 PATHWAY

The TNF-R-like CD40 molecule has been known for many years to be present on B cells and antigen presenting cells including follicular DC (*13*, *14*). Its ligand, the TNF-like CD40L is induced on virgin T cells within a few hours of their activation. This is one of the consequences of the first (TCR-mediated) signal and may be reinforced by signals via CD28. CD40L engagement on T cells leads to increased density of CD28, and later to induce ICOS, while on the APC, CD40 engagement is mandatory for further APC activation. CD40 stimulation activates the carrying APC in many functional aspects as this is emphasized for B cells. Thus the pathway is mandatory for primary APC –T cell cooperation. It also plays a prominent direct role in T-B cell cooperation because CD40 transduce signals, within B cells, that are required for their activation, maturation and Ig class switch. This is emphasized by the disorders observed in the human hyperIgM syndrome, an X-linked immunodeficiency where the CD40L molecule is non functional. While the CD40L and CD28 pathways work together in a close relationship, the outcome of the T helper response can not be completely superimposed since in contrast to CD28 stimulation, CD40L tends to raise TH1 type response. It preferentially induces IL-12 secretion from APC, the pivotal cytokine for inducing TH1.

MANY OTHER TNF–TNF-R PATHWAYS ACT TO DEVELOP AN EFFICIENT T-CELL RESPONSE

The OX40–OX40L Pathway, a Third Player in the CD28, CD154 Ball Game

OX40 is a TNF-R-like molecule that rapidly appears on virgin T cells after their activation with a CD28 cosignal (*15*). The ligand for OX40 (OX40L, gp34) belongs to the TNF-like family and appears on the surface of DC solely after they have been activated by the CD40L induced on virgin T cells. In other words, the sequence of induction is T cell CD28 - T cell OX40 and CD40L - OX40L on DC. Most CD4 blasts in the T-cell zone are $OX40^+$, and both CD28 and OX40 are responsible for the proliferation peak observed on day 7 during priming. OX40 KO mice have impaired CD4 priming, whereas mice overexpressing OX40L on DC have an accumulation of activated memory cells. In addition to CD4 proliferation/activation, OX40, together with CD28, is responsible for the induction of CXCR5 on CD4 cells, the crucial chemokine receptor that drives the CD4 cells to B-cell follicles, enabling the formation of germinal centers. OX40 KO mice do not form GC and, in mice overexpressing OX40L on DC, oversized GC are also observed. The third effect of OX40 is that it tends to develop the response toward the Th2 side, although, as with CD28, the choice is not so clear-cut and absolute, and there are still contradictory results in the literature. Finally a fourth, somewhat paradoxical, effect has been described after the detection of OX40 on inflammatory vascular cells: the pathway is required for the migration of memory T cells within inflammatory tissues, providing an explanation of the paradoxical attenuation of EAE by OX40 pathway blockade.

The CD137/4-1BB–4-1BB-L Pathway for Developing Cytotoxic T-Cell Responses

4-1BB is a TNF-R-like molecule present on activated CD4 and CD8 cells, as well as natural killer cells (*16*). Its ligand, a TNF-like molecule, 4-1BB-L, is present on APC, including DC, macrophages, and B cells. 4-1BB is a costimulatory molecule that induces IL-2 secretion, proliferation, and is required for generating cytotoxic T cells. In vivo, stimulation via 4-1BB favors cytotoxic T lymphocyte generation against both allogeneic and tumoral cells. Activation of both the CD4 and the CD8 cells are required for these effects, though the pathway seems to be more mitogenic for CD8 rather than CD4 cells. Reciprocally, it would also contribute, through its ligand, to the maturation/activation of APC. Interestingly, the pathway seems efficient in manipulating tumor and graft rejection, including graft-versus-host diseases.

The TRANCE–RANK Pathway for Obtaining Antiviral Responses

Although CD40- or CD154-deficient mice suffer from a profound defect of humoral immunity, they may still develop a relatively good response to viral antigens. The TNF-Related Activation iNduced Cytokine (TRANCE), also known as the osteoclast differentiation factor, which shares 18–28% homologies with other TNF-like factors, can be detected on T cells from lymph nodes (*17*). As

for DC, TRANCE reacts with a TNF-R-like molecule Receptor Activator of NF-κB (RANK). TRANCE strongly activates DC via RANK, inducing similar effects to the CD154-CD40 interaction. Blocking the pathway with TRANCE-Fc molecule has no effect in normal mice. However, in CD40-deficient mice, this defect produces a lack of CD4 response to lymphocytic choriomeningitis virus with no IFN-γ production. TRANCE KO mice have a defect in allogeneic proliferative responses, both in Th1 and Th2 terms. They also have blocking effects in early stages of T- and B-cell differentiation, but not on DC; and they present lymph node hypoplasia but normal spleen and Peyer's patches, which do not seem to be caused by deficient lymphocyte homing.

APRIL and BAFF, TACI and BCMA to Directly Activate B Cells

APRIL has recently been cloned as TNF-like with a strong capacity to induce proliferation of various tumoral cells. Although its ligand on tumoral cells has not yet been described, it was found to react with a B-cell and a T-cell surface molecule known for several years (*18*). These are BCMA for B Cell Maturation Antigen and TACI for Transmembrane Activator and CAML Activator, a surface molecule known to induce a transcription factor involved in T-cell activation. This is, in fact, a party of four because both BCMA and TACI were also found to react with another TNF-like receptor, BAFF (also known as TALL-1). BAFF is constitutively expressed by macrophages, DC, and activated T cells; APRIL shows greater expression on peripheral blood lymphocytes, predominating on monocytes/macrophages. However, BCMA has limited expression on $CD19^+$ resting mature B cells, and TACI has larger expression on B cells and is also present on activated CD4 and CD8 cells. BAFF and TACI trigger the same effect while binding BCMA or TACI. They are comitogenic for B cells together with BCR cross-linking. They allow survival of immature B cells that undergo negative selection. Transgenic mice overexpressing BAFF display marked B-cell hyperplasia in secondary lymphoid organs with accumulation of activated B cells with anti-DNA and other autoantibodies in their sera in addition to an increased number of memory effector T cells. BAFF may be involved in the production of autoimmune disorders. Thus, and most importantly, the four players play a crucial game for B-cell proliferation and survival but do not induce any class switch; the pathway has probably been captured in part in favor of tumor cells.

MORE ON B-CELL ACTIVATION AND THE SHAPING OF MEMORY CELLS, THE CD27–CD70 PATHWAY

CD27, a TNF-R-like molecule present on naive T cells, greatly increases while T cells activate but irreversibly disappears in the later stages of T-cell differentiation (*19*). $CD27^+$ B cells have a mature phenotype. CD70, the recognized ligand for CD27, shows restricted expression on activated B and T cells. It is therefore is likely that this couple works for T-B cooperation but also for T-T and B-B cooperation. In vitro, CD27 is slightly comitogenic for B cells but induces their differentiation in plasmocytes. CD27 KO mice have a defect in the expansion of naive T cells but not in their maturation; however, they lack memory, particularly CD8 cytotoxic T lymphocytes.

MORE ON Th2 ACTIVATION: CD30

CD30 was identified as an antigen typical of Reed-Sternberg cells, a TNF-R-like molecule also found on subsets of activated T and B cells (*20*). It is quickly and massively shed as a soluble factor from the cell surface. Although CD30 KO mice have normal Th2 differentiation, it is believed that in inflammatory conditions, soluble CD30 would favor a Th2 response to counteract Th1 differentiation in an attempt to compensate for Th1-induced disorders, such as rheumatoid arthritis.

A FEW WORDS ABOUT OTHER MOLECULES

They are many more T-cell surface molecules, not related to the two large families and that seem to transmit, or contribute to, a coactivation signal. It is not surprising that so many T-cell surface molecules are aimed at modulating the activation of the carrying cell: T cells are circulating cells that operate in many different conditions and that must take into account the numerous specificities of their environment and the context in which the immune reaction has to occur. As for the two major families involved in the heart of T-cell activation, namely the APC-T cell cooperation, they have intimately mixed their destinies, with love and hate, for the sake of sophisticated, accurate immune responses.

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