Immunological tolerance: **Danger – pathogen on the premises!**

Charles A. Janeway Jr*, Christopher C. Goodnow† and Ruslan Medzhitov*

Recent results show that immune responses can be induced in neonatal mice. Do they really refute the traditional view that the ability to discriminate between 'self' and 'non-self' is a fundamental property of the immune system?

Addresses: *Section of Immunobiology, Yale University School of Medicine, New Haven, Connecticut 06510, USA. †Department of Microbiology and Immunology, Stanford University Medical School, Stanford, California 94305-5428, USA.

Current Biology 1996, Vol 6 No 5:519–522

The March 22 issue of *Science* carried three articles [1–3] that claim to challenge a key principle of immunology, called self–non-self discrimination, by examining neonatal tolerance. These papers demonstrate that newborn mice can make immune responses if the antigen is appropriately presented. Is this a refutation of self–non-self discrimination as the authors believe? And does it prove the 'danger theory' of Polly Matzinger, a leading proponent of this idea and the senior author of the first of the three articles? We shall argue that self–non-self discrimination is alive and well, and that the 'danger signals' that alert the immune system are co-stimulatory molecules and cytokines induced by pathogens recognized by the innate immune system.

In 1945, Ray Owen reported that fraternal twin cattle born from a single placenta, or 'freemartin' cattle, had exchanged their blood cell precursors *in utero*. As a consequence, the immune system of these cattle appeared to tolerate the continual presence of genetically foreign fraternal erythrocytes, rather than reject them as would normally occur following a mismatched blood transfusion. This striking observation prompted Mac Burnet and Frank Fenner to propose in 1949 that, during intrauterine life, the immune system learns to recognize 'self' antigens by a process of actively acquired self-tolerance.

In the 1950s, Peter Medawar and colleagues [4] showed that freemartin cattle had indeed acquired tolerance to each others' tissue antigens that was so profound that it prevented the normally vigorous rejection of skin grafts between such genetically dissimilar individuals. Subsequently, Medawar and colleagues repeated this experiment in neonatal mice by showing that infusing spleen and bone marrow cells from mouse strain A into neonatal mice of strain B induced a state of immunological tolerance to strain A skin grafts; this is called neonatal tolerance. Subsequent studies showed that the grafted skin retained antigens that could be recognized by strain B lymphocytes, as infusing lymphocytes from a strain B mouse immune to a strain A mouse led to skin graft rejection. Surprisingly, normal strain B lymphocytes would not cause graft rejection, even though normal strain B mice can reject strain A skin [4]. These experiments can now be interpreted to mean that tolerance requires chimerism, the persistence of cells from the donor in the circulation of the recipient; when donor cells are removed, the graft is rejected. This also indicates that tolerance is an ongoing process in adult mice.

Many experiments in the 1950s, 1960s, and 1970s argued against Burnet and Medawar's simple notion that tolerance was acquired because of a special state of immune cells *in utero* and in neonates. In particular, immune responses to a variety of self tissues could be induced if the self tissue antigens were presented in emulsions mixed with bacteria (adjuvants). Moreover, tolerance in the humoral, or antibody-mediated, arm of the immune system could be induced to foreign antigens in adult animals if the foreign antigens were given in the right dose and form, and in the complete absence of bacterial contaminants such as endotoxin.

These findings did not challenge the central notion that tolerance to self is an actively acquired process, but highlighted the fact that other factors, such as the amount of antigen and association with 'co-stimuli', might be more important than the time of antigen administration in determining the outcome of introducing a foreign protein into an animal. Nevertheless, similar states of tolerance to skin grafts were difficult to induce in adult mice in the cell-mediated arm of the immune system, reinforcing the idea that the neonate is unique in its failure to mount a graft rejection response.

This idea has now been conclusively refuted by Polly Matzinger and co-workers [1], who have shown that neonatal mice have a small number of mature peripheral lymphocytes that can be immunized if an appropriate stimulus is used. Two other papers [2,3] appearing in the same issue of *Science* also document immune competence in neonatal mice to antigens administered in appropriate form. This has been interpreted by Matzinger to mean that self–non-self discrimination is not a key feature of immune responses, and that a mysterious 'danger signal' is the true key to understanding immune system function.

Time to discard self–non-self discrimination?

Matzinger argues that her group's experiments challenge a central tenet of Burnet's [5] clonal selection hypothesis of

adaptive immunity, which is that developing lymphocytes with self-reactive receptors are eliminated before they achieve functional maturity. In Matzinger's case, this would apply to T lymphocytes, the mediators of graft rejection. Matzinger and colleagues [1] showed that neonatal mice can be rendered tolerant using the regimen employed by Medawar [4]; however, they made two other findings that argued against Medawar's interpretation of his results.

First, Matzinger and colleagues [1] were able to induce a consistent immune response in neonatal mice if they used dendritic cells as the immunogen instead of the spleen and bone marrow cells used by Medawar. Dendritic cells are a rare (~1 %) but highly potent stimulator of the cytotoxic T cells whose response Matzinger and colleagues measured. Second, they were able to induce tolerance in adult mice if the innoculum was large enough ($1–10 \times 10^8$ cells). This proves that neonatal mice have competent T cells that can respond if the antigen is presented in the proper context. Does it also mean that self–non-self discrimination is not a fundamental principle of adaptive immunity? We argue that it does not.

That self–non-self discrimination is a fundamental property of the immune system is in fact integral to the way Matzinger and colleagues conduct their own experiments, as well as to virtually all experiments in immunology. Matzinger and colleagues used a well-characterized system to prove that acquired tolerance in neonatal mice is not due to clonal deletion of developing T cells, but to inappropriate presentation of the foreign antigen that they used — an antigen called H-Y because it is expressed by cells of male mice but not those of female mice. Male mice do not respond to H-Y because all their T cells are eliminated in the thymus during development [6].

Ironically, Matzinger herself published an important paper [7] showing for the first time that dendritic cells are the most potent cell type for eliminating self-reactive T cells during intrathymic development. The process of self tolerance is an ongoing one throughout life; immature T cells can be made tolerant by potent antigen-presenting cells, whereas mature T cells can be activated by the same cells. Therefore, self tolerance to important antigens is guaranteed by clonal deletion before T cells achieve functional maturity in the thymus. The clear implication of neonatal tolerance is that it is not the age of the mouse but the maturity of the T cell that determines its susceptibility to elimination. We conclude that the immune system does have the property of self–non-self discrimination.

How does the immune system decide when to respond?

The main difference between the original experiments of Medawar and the recent results of Matzinger and colleagues is that the latter used enriched dendritic cells to immunize the mouse. Dendritic cells are rich in molecules involved in T-cell activation called co-stimulatory molecules. The notion that T cells require a signal in addition to ligation of their receptors has developed through several steps. In the early 1970s, Bretscher and Cohn [8] contended that lymphocyte activation would require two signals, each delivered by an antigen-specific cell, in order to explain the requirement for recognition of two distinct epitopes on an antigen to get antibody production. Subsequently, Lafferty and Cunningham [9] argued that T-cell activation also required two signals, one antigen-specific and the other a 'co-stimulatory signal' that was delivered by passenger leukocytes in the grafting model they studied.

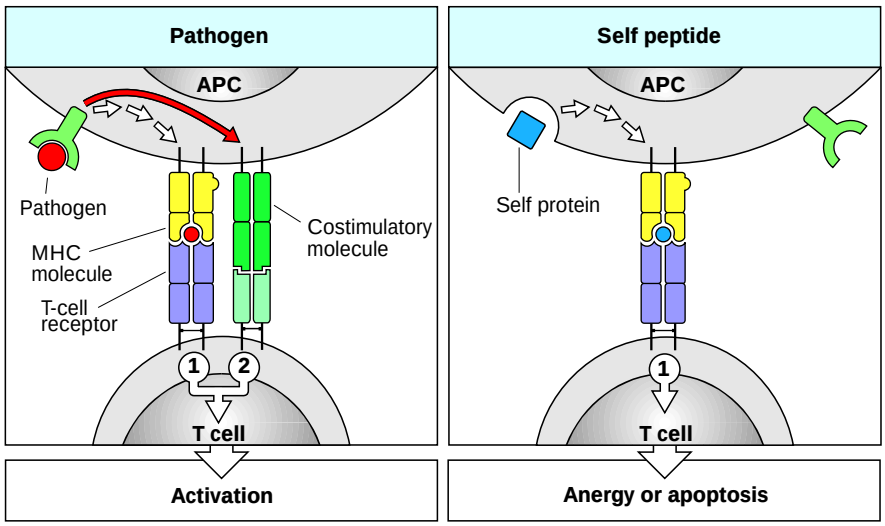
Subsequent studies have validated the Lafferty–Cunningham hypothesis [10]. In an important extension of this idea, Liu and Janeway [11] showed that both signals need to be delivered by the same cell for an optimal T-cell response to occur (Fig. 1). This finding is implicit in many studies, as for instance in the ability of male dendritic cells to produce immunity to H-Y in neonatal female mice [1]. Dendritic cells present antigens, including the male-derived H-Y, on a cell that is highly co-stimulatory [12]. Other cells, such as macrophages and B cells, also present H-Y, but do not express co-stimulatory molecules constitutively, and thus tend to induce tolerance rather than immunity. Such cells can be induced to express co-stimulatory molecules, and this can be used to search for clues to the recognition events that are crucial to understanding the 'danger signal' proposed by Matzinger [13].

What signifies danger for a vertebrate?

Matzinger [13] has proposed that the immune system is not regulated by self–non-self discrimination, but rather by danger signals. What represents danger to the vertebrate? We have argued in the past [6,14,15] that danger comes in the form of infection in vertebrates, and that there must be a system by which such danger indications are translated into signals that inform the T cells to make a response. We will briefly explain our thinking.

Vertebrates that lack the capacity to mount adaptive immune responses because of genetic defects in one or another arm of adaptive immunity normally die of recurrent or overwhelming infection. Such individuals only became obvious after antibiotics were introduced in the 1930s and 1940s; before that time, it is likely that all such individuals died of infection, as did many normal individuals. For instance, the influenza pandemic in 1919 killed an estimated 28 000 000 people around the world. Thus, the main selective pressure on adaptive immunity was exerted in relation to the ability of an individual to mount an effective response to microbial pathogens. Other manifestations of the immune response, such as graft rejection,

Figure 1



The difference between tolerance and immunity is illustrated here for CD4 T cells. Pathogens (left panel) are bound by special receptors on antigen-presenting cells (APCs), which are specific for pathogen-associated molecular patterns. Such receptors are involved in two processes. First, they allow efficient uptake and processing of the pathogen-associated antigens, which are expressed on the cell surface bound to a major histocompatibility complex (MHC) class II molecule. Second, they induce (red arrow) expression of co-stimulatory signals, such as B7 molecules, by the APC. The these two signals (1 and 2, left panel) activate a CD4 T cell bearing an appropriate T-cell receptor. Self proteins (right panel) are not recognized by the 'pattern' receptors, and thus do not induce expression of co-stimulatory molecules on the APC and deliver only signal 1 to the T cell, which induces tolerance as either anergy or apoptosis. The co-receptor CD4, an MHC class II-binding molecule on the T cell, is also involved in signal 1 but is omitted for simplicity. In addition to the signals shown here, pathogens also induce production by the APCs of inflammatory and effector cytokines.

tumor immunity and autoimmune diseases, only emerged in this century, and thus have played no role in selecting the response parameters of the immune system. Therefore, one must try to understand the adaptive immune response mainly in terms of its ability to recognize and respond appropriately to microbial pathogens.

The antigen receptors of T cells and B cells are generated at random by rearrangement of gene segments, and these are selected for fitness during T-cell and B-cell maturation in specialized microenvironments [6,16]. The specificity of these receptors is crucial to self–non-self discrimination, but these receptors cannot tell the T cell what response to make upon encountering antigen after they complete their maturation and emigrate to the periphery. Should they respond by becoming activated, as in Matzinger's experiment, or by becoming tolerant as in Medawar's original studies? How does such a system make the right choice in the majority of cases?

We believe the immune system does so by using signals induced by recognition of conserved patterns of molecules made by microbial pathogens but not by vertebrate cells. It has been shown that such molecules expressed by microbes can stimulate expression of co-stimulatory molecules (Fig. 1) [17]. The receptors for this signalling are known in some cases: the scavenger receptor expressed by macrophages and dendritic cells is one such case, but there are many others. It is interesting that the other two papers in the same issue of *Science* used dead bacteria [2] or live virus [3] to show that neonatal T cells can make an appropriate response.

The properties of these pattern-recognition receptors are completely different from those that mediate self–non-self discrimination in the adaptive immune response. They are encoded by non-rearranging genes and are expressed by all cells of a certain type. They are directly linked to specific responses, inducing expression of co-stimulatory molecules and cytokines that condition the nature of the subsequent T-cell response. T cells, once empowered to make a response, pass on that message to the B cells with which they interact, instructing such cells to make antibody by expression of a B-cell co-stimulatory molecule called CD40 ligand. Patients who cannot make CD40 ligand are profoundly immunodeficient [18].

How to test the danger model directly

If one argues that self–non-self recognition does play a key role in adaptive immunity, how could one test for it? This could be accomplished by forming a chimeric mouse, all of whose tissues are male but which is unable to produce T cells because of deletion of its T-cell receptor α chain genes. The T cells in this mouse would derive from bone marrow cells of a female mouse with intact T-cell receptor α chain genes. The danger signal would be provided by male dendritic cells, just as in the experiments by Matzinger and colleagues [1]. This complicated test is necessary, as one might not detect male-specific killing if the killer cell itself could express H-Y. We would predict that such mice will not mount an immune response to H-Y, because self tolerance should be dominant; Matzinger's hypothesis predicts that such mice should make a response, as they have received the relevant antigen on

cells that carry a danger signal in the form of effective co-stimulators. May the best mouse win!

Conclusions

The papers of Matzinger and the other authors [1–3] have redefined the immune status of the neonatal mouse; no longer can we blithely speak of a window of tolerizability in the neonate. They have not, however, upset current paradigms of immunology, as the authors would like to suggest [19,20]. Rather, they can be readily incorporated into the mainstream of immunology. The receptors of T and B lymphocytes are carefully scrutinized during development for their ability to recognize self antigens, and those that do are eliminated during their early development in the appropriate microenvironment, or are removed or silenced upon emigration to the periphery by encountering many antigens that were not met during intrathymic development on cells that lack co-stimulatory molecules. Such encounters do not trigger a response because no co-stimulators are present.

What really gets the attention of mature T cells is presentation of specific foreign peptides in the context of an appropriate co-stimulatory signal. Dendritic cells isolated from lymphoid tissues express such co-stimulatory signals constitutively [1,12], and these have been shown to be inducible on B cells and macrophages on encounter with microbial molecules such as lipopolysaccharide, mannans, glucans, bacterial DNA, double-stranded RNA and other substances that are present in pathogens but not in vertebrates [17]. Thus, the real danger signal that is critical for the effective induction of an adaptive immune response appears to be mediated by invariant receptors recognizing pathogen-associated molecular patterns. The real danger signal is transmitted by the presence of a pathogen, which creates a signal telling the body "Danger — pathogen on the premises!"

References

1. Ridge JP, Fuchs EJ, Matzinger P: Neonatal tolerance revisited: Turning on newborn T cells with dendritic cells. *Science* 1996, 271:1723–1726.
2. Forsthuber T, Yip HC, Lehmann PV: Induction of Th1 and Th2 immunity in neonatal mice. *Science* 1996, 271:1728–1730.
3. Sarzotti M, Robbins DS, Hoffman PM: Induction of protective CTL responses in newborn mice by a murine retrovirus. *Science* 1996, 271:1726–1728.
4. Billingham RE, Brent L, Medawar PB: Actively acquired tolerance of foreign cells. *Nature* 1953, 172:603–606.
5. Burnet FM: *The Clonal Selection Theory of Immunity*. Nashville, Tennessee: Vanderbilt University Press; 1959.
6. Goodnow CC: Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires. *Proc Natl Acad Sci USA* 1996, 93:2264–2271.
7. Matzinger P, Guerder S: Does T cell tolerance require a dedicated antigen-presenting cell? *Nature* 1989, 338:74–76.
8. Bretscher P, Cohn M: A theory of self-nonself discrimination: paralysis and induction involve the recognition of one and two determinants on an antigen, respectively. *Science* 1970, 169:1042–1049.
9. Lafferty KJ, Cunningham AJ: A new analysis of allogeneic interactions. *Aust J Exp Biol Med Sci* 1975, 53:27–42.
10. Janeway CA Jr, Bottomly K: Signals and signs for lymphocyte responses. *Cell* 1994, 76:275–285.
11. Liu Y, Janeway CA Jr: Cells that present both specific ligand and costimulatory activity are the most efficient inducers of clonal expansion of normal CD4 T cells. *Proc Natl Acad Sci USA* 1992, 89:3845–3949.
12. Steinman RM: The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 1991, 9:271–296.
13. Matzinger P: Tolerance, danger, and the extended family. *Annu Rev Immunol* 1994, 12:991–1045.
14. Janeway CA Jr: Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 1989, 54:1–13.
15. Janeway CA Jr: The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today* 1992, 13:11–16.
16. von Boehmer H: Positive selection of lymphocytes. *Cell* 1996, 76:219–228.
17. Liu Y, Janeway CA Jr: Microbial induction of costimulatory activity for CD4 T cell growth. *Int Immunol* 1991, 3:323–332.
18. Banchereau J, Bazan F, Blanchard D, Briere F, Galizzi JP, van Kooten C, Liu YJ, Rousset F, Saeland S: The CD40 antigen and its ligand. *Annu Rev Immunol* 1994, 12:881–922.
19. Pennisi E: Teetering on the brink of danger. *Science* 1996, 271:1665–1667.
20. Johnson G: Findings pose challenge to immunology's central tenet. *NY Times* March 26, 1996:C1–3.