

Natural adjuvants: Endogenous activators of dendritic cells

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Dendritic cells, the most potent antigen-presenting cells, need to be activated before they can function to initiate an immune response. We report here that, in the absence of any foreign substances, dendritic cells can be activated by endogenous signals received from cells that are stressed, virally infected or killed necrotically, but not by healthy cells or those dying apoptotically. Injected *in vivo* with an antigen, the endogenous activating substances can function as natural adjuvants to stimulate a primary immune response, and they may represent the natural initiators of transplant rejection, spontaneous tumor rejection, and some forms of autoimmunity.

The stimuli that activate dendritic cells^{1–4} (DCs), and thereby initiate immune responses, are the subjects of intense study, not only because they are crucial for the production of more effective adjuvants for use in vaccines, but also because they are essential to the initiation of transplant rejection, tumor immunity, autoimmunity and the immunobiology of the maternal–fetal interface.

There are now two lines of thought concerning the nature of DC activating signals. The first suggests that cells involved in innate immunity, like those mediating adaptive immunity, are focused outward towards the recognition of foreign entities. For DCs, such recognition is thought to occur through a set of pattern recognition receptors that have evolved to recognize conserved molecules on bacteria and other evolutionarily distant organisms^{5,6}. In favor of this view are findings that DCs can be activated by bacterial lipopolysaccharide (LPS) and that they have receptors for the mannans of yeasts^{7,8}.

Alternatively, it has been proposed that DCs, like platelets and endothelial cells⁹, may be focused inward, and respond to endogenous signals released by cells undergoing stress, damage or necrotic death^{10,11}. Favoring this view is evidence that DCs can be activated by secondary mediators of inflammation, such as tumor necrosis factor- α and activated T cells¹². However, these are features of an immune response that require activated antigen-presenting cells (APCs) for their inception and are thus likely to be part of a positive feedback loop, rather than the initiators of immunity.

To search for early, non-immune, endogenous DC activators, we used an *in vitro* system in which activation of resting DCs can be assessed by changes in surface markers involved in their function as APCs, such as the antigen-presenting molecules MHC class I and II, the co-stimulatory molecules B7.1 and B7.2, and the receptor for T-cell signals CD40. To determine whether such activation is immunologically functional, we used *in vitro* and *in vivo* tests of immunity. We found that direct stress, signals from necrotic cells, and a virally induced cytokine were able to activate resting DCs, whereas healthy cells or those dying normal programmed cell deaths did not.

Activation without addition of foreign substances

We grew DCs from bone marrow precursors, modifying a pub-

lished method¹³, to manipulate the cells as little as possible. After 6–7 days, the cultured DCs had a relatively unactivated phenotype, expressing low levels of activation markers in a manner that resembled the immature, resting DCs serving as peripheral sentinels *in vivo*¹⁴ (Fig. 1a). However, they had reached a level of maturation wherein they were able to upregulate B7.1 and B7.2, MHC class II and CD40, and achieve a classical activated/mature immunostimulatory phenotype in response to the powerful bacterial activator, LPS. (Fig. 1b). To determine whether direct stress would have an effect, as it does on platelets and some plant cells¹⁵, we pipetted the DCs to dissociate any cell interactions, and placed them into fresh plates overnight. Such manipulation induced activation equivalent to that induced by LPS (Fig. 1c). We do not yet know if the activation is due to direct stress to the DCs themselves, perhaps from loss of contact with fibroblasts or extracellular matrix molecules laid on the plastic, or to damage to other cells in the culture. In either case, it does not require any bacterial or otherwise foreign substances. LPS given to the DCs at the same time as the transfer, was only slightly additive (Fig. 1d), indicating that the transferred DCs were fully activated and did not respond better to further stimuli.

Activation by pre-packaged signals

In the transfer experiment described above, the DCs were directly manipulated. *In vivo*, however, an infectious or non-infectious insult can damage the cells of a tissue without necessarily involving the DCs themselves. We therefore tested whether DCs can respond to signals from damaged cells that do not belong to the immune system by adding to the resting DCs (Fig. 2a) syngeneic fibroblasts that had been rendered necrotic (by repeated freezing and thawing) or apoptotic (by ceramide treatment), or had been left to grow in log phase. The addition of necrotic cells induced upregulation of MHC and co-stimulatory molecules (Fig. 2d), indicating that DCs can respond to pre-existing signals from neighboring cells made available upon the loss of membrane integrity during necrosis. The addition of equal numbers of healthy syngeneic fibroblasts had no stimulatory effect on the resting DCs (Fig. 2b). Instead, the number of spontaneously activated DCs decreased, indicating that the fibroblasts contributed to the health of the culture, perhaps by secreting cy-

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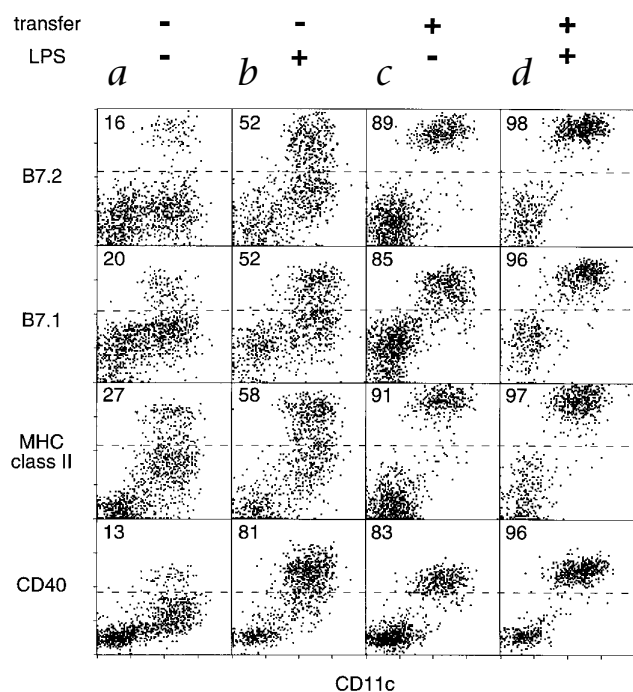


Fig. 1 Resting dendritic cells can be activated by mechanical manipulation in the absence of foreign substances. Bone marrow-derived B10.SCID DCs were grown for 6 d and then either collected and transferred into fresh wells (transfer +) or left in the starting wells (transfer -), incubated for an additional 24 h in the presence (+) or absence (-) of 1 μ g/ml LPS, then collected and stained for the DC-specific marker CD11c and activation markers (left margin). Upper left corners, percentages of CD11c-positive cells that are positive for the activation markers.

tokines that keep DCs in a resting state. Similarly, apoptotic fibroblasts induced no activation (Fig. 2c), and except for B7.1, which was sometimes slightly increased by apoptotic cells (not shown), the levels of co-stimulatory molecules were often lower than in control cultures. This stabilizing effect, which was also seen with cells driven into apoptosis by serum starvation after treatment with mitomycin C (not shown), may also be due to cytokines released by fibroblasts during the apoptotic process, either as a last sign of vitality or as an active anti-inflammatory reaction^{16,17}. Thus pathological and harmful death, like necrosis, exposes signals that activate DCs, whereas normal apoptotic cell death does not.

Resting DCs capture both apoptotic and necrotic cells

Previous studies¹⁸⁻²⁰ have shown that DCs can endocytose apoptotic cells and that antigens captured in this manner can be displayed on the surface. Those early studies, however, did not discriminate between resting and activated DCs. We therefore tested the endocytic capacity of cultured resting C57BL/6-recombination activation gene (Rag) knockout (H-2^b) DCs by adding either necrotic or apoptotic BALB/c (H-2^d) fibroblasts previously stained with the green intracellular dye CFSE. After the incubation, we stained the mixed cultures with monoclonal antibodies against CD11c and H-2D^d (the latter cannot penetrate the DC membrane and thus labels only the free BALB/c fibroblasts). Using these three reagents, we could discriminate free-floating fibroblasts (green, H-2D^{d+}) from those that had been engulfed (green, CD11c⁺, H-2D^{d+}) and those that were simply attached to DCs (green, CD11c⁺, H-2D^{d+}) (Fig. 3b and c). The rest-

ing DCs (Fig. 3a) picked up necrotic and apoptotic cells to the same extent (Fig. 3e and f). Thus, resting DCs are indeed able to capture antigens from apoptotic cells, but in conditions in which the number of apoptotic cells is not overwhelming (two apoptotic cells for each DC), and when both the DCs and the fibroblasts are handled as gently as possible, this does not induce activation or the upregulation of MHC or of co-stimulatory molecules (Fig. 2c). Presentation in this case would most likely lead to induction of T-cell tolerance²¹.

Functional consequences of activation

To determine whether the phenotypic activation induced by endogenous signals was accompanied by functional activation, we tested the ability of our DCs to present antigen to Marilyn, an IA^b-restricted, CD4⁺ T-helper cell type 1 (Th1) clone specific for the male antigen H-Y. Because H-Y is expressed only on male cells, we could assess the ability of DCs to process and present their own intrinsic cellular antigens (using male DCs) or to process and present antigens captured from other cells (using male fibroblasts and female DCs). We first tested male DCs, which carry their own H-Y antigen, and found that this CD4⁺ T-cell clone responded very well to male DCs that had been co-cultured with necrotic male fibroblasts, but not to resting male DCs or to those that had been co-cultured with apoptotic male fibroblasts (Fig. 4a).

To determine whether the enhanced function of the activated DCs was simply due to increased co-stimulatory capacity or whether they were also able to process exogenous antigens, we added necrotic or apoptotic male fibroblasts to resting female DCs, which do not express H-Y. Co-culture with necrotic, but not apoptotic, male fibroblasts stimulated the female DCs to process the male antigen and induce strong T-cell proliferation (Fig. 4b). Thus, resting DCs can be induced to upregulate expression of co-stimulatory molecules and to capture, process, and present antigens from dying necrotic cells.

In responses against foreign transplants, activated donor DCs induce CTL immunity against the donor antigens. It is commonly believed, however, that T-helper responses to foreign tissues are due to the 'indirect' pathway, in which host APCs present the donor antigens^{22,23}. To determine whether endogenous signals can induce DCs to present their own intrinsic antigens to CD4 T-helper cells by the direct pathway, we cultured resting male DCs with female fibroblasts, thus supplying activation signals but no additional source of H-Y. Necrotic female fibroblasts were indeed able to induce male DCs to present their own H-Y antigens, though not to the same extent as DCs that had been activated by necrotic male fibroblasts (Fig. 4c). This indicates that signals from necrotic cells may activate both the direct and indirect pathways of antigen presentation in DCs, though, for MHC class II, the indirect pathway seems somewhat more efficient.

DCs, activated by the manipulations necessary to prepare them for the T cell stimulation, were also able to present antigens that they had previously captured from apoptotic cells (Fig. 4b and c), although the effect was blunted by the brevity of the assay. This has important implications for autoimmune diseases, in which extrinsic inducers of cell death (such as pathogens) or intrinsic mutations in the genes controlling apoptosis may lead to recurrent or persisting activation of local APCs.

Activation by inducible signals

If DC activating signals came only in 'pre-packaged' form,

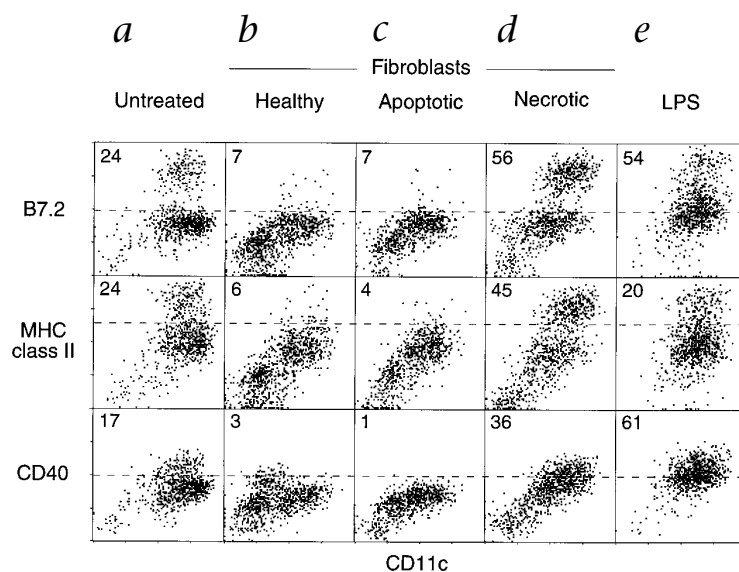


Fig. 2 Resting dendritic cells can be activated by co-culture with necrotic syngeneic cells. B10.SCID bone marrow-derived DCs were grown for 6 d; then stimuli (above plots) were added to the wells for a further 24 h of culture. Cells were then collected and stained for the DC-specific marker CD11c and activation markers (left margin). **a**, Untreated. **b**, 1×10^6 healthy B10.SCID fibroblasts in log phase growth. **c**, Ceramide-induced apoptotic fibroblasts. **d**, Frozen-thawed fibroblasts. **e**, 1 μ g/ml LPS. Upper left corners, percentages of CD11c-positive cells that are positive for the activation markers.

viruses that induce apoptosis would escape immunity. To explore the idea that virally infected cells might produce signals that alert their neighboring DCs, even in the absence of necrotic death, we tested the stimulatory capacity of interferon (IFN)- α , an antiviral cytokine made by many types of virally infected cells^{24,25} (including DCs; ref. 26) that has immunomodulatory functions²⁷ and acts in synergy *in vitro* with tumor necrosis factor- α in the maturation of DCs (ref. 28). To determine whether IFN- α could act alone as a DC-activating signal, we stimulated resting DCs with several different doses of recombinant mouse IFN- α . At doses approximating those expected to occur locally at the site of a virus infection²⁹, IFN- α acted as a substantial stimulus to resting DCs (Fig. 5) as well as to DCs that had been simultaneously given apoptotic fibroblasts (not shown). Of note, the activation pattern resembled neither that seen with necrotic cells nor that seen with LPS. IFN- α stimulated greater increases in B7.2 than in B7.1, whereas LPS and cells that had been frozen and thawed upregulated both of these co-stimulatory molecules similarly. This indicates that different types of signals might stimulate DCs in different ways, perhaps to elicit different functions.

Priming naive T cells

In vitro, our resting DCs responded to endogenous signals by up-regulating co-stimulatory molecules and gaining the capacity to activate a T-cell clone. There are, however, two shortcomings with this system. These are quite immature DCs grown in artificial culture conditions. Moreover, any T-cell clone comprises previously activated cells and therefore does not test whether activated DCs have become true professional APCs, able to stimulate naive T cells³⁰. We therefore tested the activating properties of endogenous signals in a primary T cell response against ovalbumin (OVA) *in vivo*. We injected 10 μ g OVA mixed either with

apoptotic fibroblasts, fibroblasts that had been frozen and thawed, IFN- α , complete Freund's adjuvant (a positive control), or phosphate-buffered saline (PBS; a negative control for non-specific damage from the injection), subcutaneously at the base of the tail into naive BALB/c mice. To avoid *in vitro* culture effects, we also injected OVA mixed with freshly isolated and damaged blood vessels. We chose blood vessels, rather than a solid organ, because they are involved in the early events of blood clotting and are likely to be injured in most cases of tissue damage. Then, 7–8 days later, we challenged the mice intradermally in the foot with 10 μ g OVA, and 48 hours later we measured the degree of local swelling resulting from the delayed-type hypersensitivity response. Both types of necrotic cells and IFN- α were excellent adjuvants for the primary anti-OVA delayed-type hypersensitivity, although they were slightly less potent than complete Freund's adjuvant, one of the strongest adjuvants known (Fig. 6). Apoptotic cells were extremely inefficient, and the low level of priming they did achieve might have been due to small numbers of cells inevitably damaged in the preparation. Thus, necrotic cells were able to activate resting DCs *in vitro* and to stimulate a primary immune response *in vivo*, whereas apoptotic cells did not.

A study using a tumor that is highly immunogenic when given as an injection of live cells showed that injections of apoptotic tumor cells did not immunize³¹. Another study, also using a highly immunogenic tumor, found that apoptotic tumor cells were 40-fold less immuno-

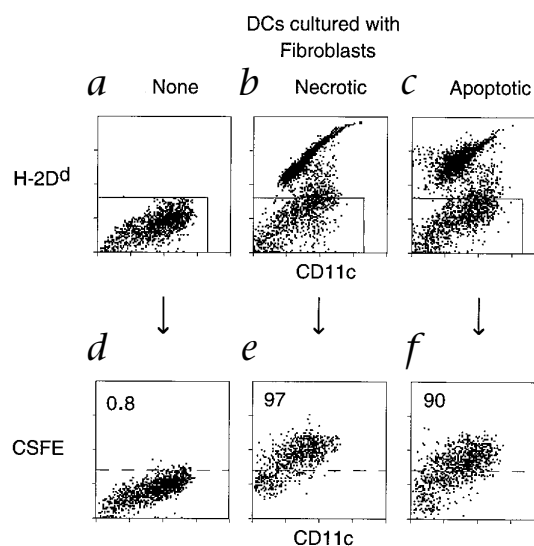


Fig. 3 Resting dendritic cells are able to engulf necrotic as well as apoptotic fibroblasts. C57BL/6-Rag knockout bone marrow-derived DCs were grown for 7 d and then incubated for 24 h with necrotic or apoptotic BALB/c fibroblasts that had been stained with the intracellular dye CFSE before being killed. **a–c**, Entire cohort of co-cultured cells. Free-floating fibroblasts stain for H-2D^d and not CD11c. Fibroblast/DC aggregates stain for both CD11c and H-2D^d. Free-floating DCs and those that have engulfed fibroblasts stain for CD11c and not for H-2D^d. **d–f**, CFSE staining of DCs that were negative for H-2D^d. DCs that have engulfed the BALB/c fibroblasts are positive for CFSE. Upper left corners, percentages of CD11c-positive cells that are positive for CFSE.

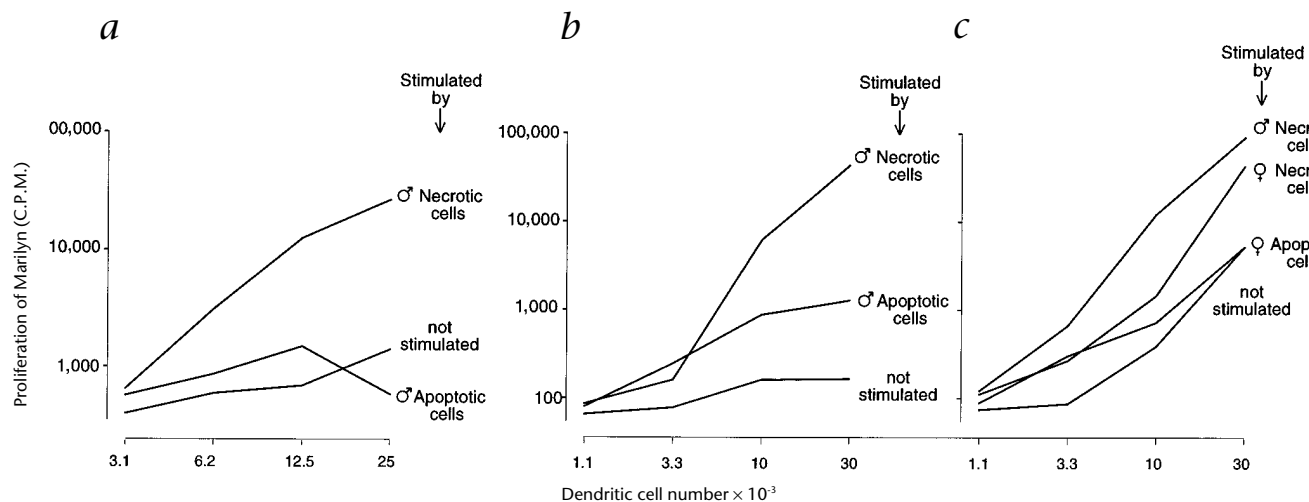


Fig. 4 Functional activation of dendritic cells. B10.SCID bone marrow-derived DCs were grown for 6 d and then co-cultured with male or female B10.SCID fibroblasts for 40 h. They were then collected, washed, irradiated and used in titrated numbers as APCs to stimulate proliferation of Marilyn, an anti-H-Y CD4 T-cell clone. Each numbered point on the horizontal axis is a point in the experimental titration. Data represent two experiments. **a**, Experiment 1: Male DCs. **b**, Experiment 2a: Female DCs. **c**, Experiment 2b: Male DCs.

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genic than live ones³². Both studies found, however, that necrotic cells that had been frozen and thawed did not immunize at all. There are many differences between those systems and ours, in antigenic stability, route of administration and so on. One likely difference concerns signal stability in a necrotic environment replete with proteases and other destructive enzymes. If DC-activating signals are highly labile, they may be

rapidly degraded. This is not normally a problem *in vivo*, where DCs are widely distributed and likely to be in close proximity to any dying cell. To minimize potential signal degradation, we were careful to keep our 'frozen-thawed' cells well-frozen until immediately before injection, at which time we allowed them to quickly reach room temperature.

Discussion

Resting DCs thus seem to respond to both pre-packaged and inducible signals from damaged cells in the absence of any exogenous 'foreign' substances. They can capture both necrotic and apoptotic cells, but the latter do not activate them to become co-stimulatory. We envisage several ways in which these properties can be combined to elicit different DC functions.

First, unstimulated DCs might continuously capture living cell exosomes³³ or apoptotic bodies, and present the captured antigens without co-stimulation to induce tolerance of autoreactive T cells²¹. This might be an especially important function for CD4⁺ T cells, which cannot be tolerized directly by MHC class II-negative tissues, for CD8 T cells specific for tissue-specific antigens expressed by cells with low levels of MHC class I (ref. 34), and for tissues that are not patrolled by naive T cells³⁵.

Second, DCs activated by prepackaged signals from necrotic cells can stimulate immune responses to antigens captured from those cells and to other local antigens, such as extracellular bacteria, toxins and so on, that might be causing the damage. This would be the basis of protective immune responses to environmental pathogens.

Third, when virally infected cells die apoptotically, local DCs will nevertheless capture the apoptotic bodies and, if stimulated by IFN- α (Fig. 5), heat shock proteins³⁶ and so on, the DCs will present the captured antigens to initiate an immune response. In this way, a virus or other pathogen cannot avoid immunity simply by inducing apoptosis in its target cell. To escape totally, the invading organism must either be harmless or it must inhibit all cellular alarm responses to infection or damage.

Fourth, the finding that cellular alarm signals activate resting DCs supports the initial assumption of the 'danger' model^{10,11},

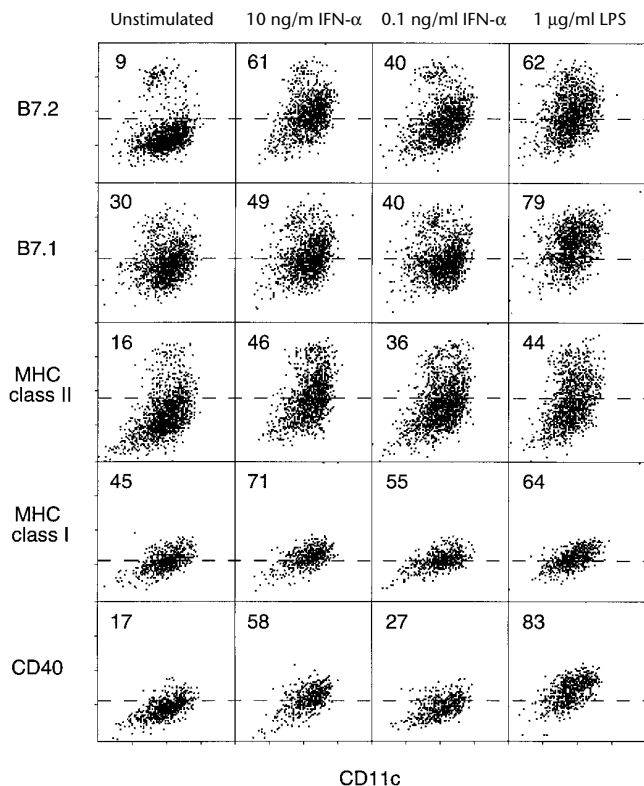


Fig. 5 IFN- α activates dendritic cells. B6 Rag knockout bone marrow-derived DCs were grown for 6 d, stimulated for 24 h, then collected and stained for the DC-specific marker CD11c and activation markers (left margin).

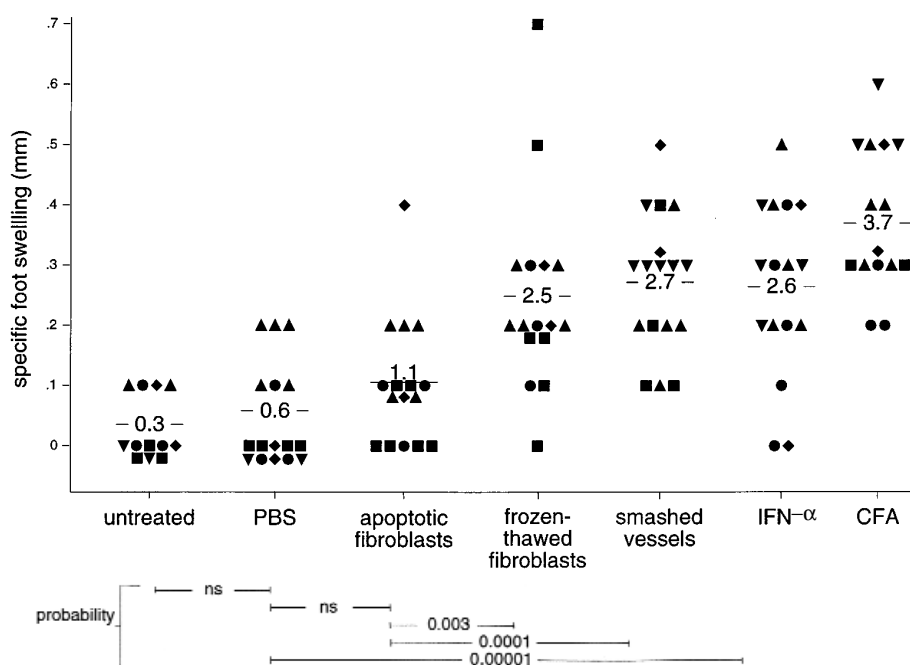


Fig. 6 Priming naive T cells. BALB/c mice ($n = 108$) were injected subcutaneously with OVA in PBS mixed with stimuli (horizontal axis), then 7–8 d later were challenged with OVA injected intradermally into the foot. Foot diameter was measured 48 h later (delayed-type hypersensitivity assay). Data represent challenged minus unchallenged foot sizes; numbers between symbols (between thin lines), averages. Each point represents a single mouse; each symbol, a different experiment. Below, statistical significance: Student's *t*-test (ns, not significant). CFA, complete Freund's adjuvant.

which envisions that immune responses can be triggered by signals coming from damaged cells. This has important theoretical and practical implications. There is an emerging consensus, pioneered by Janeway⁵, that the evolutionarily ancient 'innate' immune system is essential to the initiation of adaptive immunity, and that cells of the innate immune system are not constitutively active but are activated through pattern recognition receptors that recognize evolutionarily distant pathogens⁶. This is an important first step away from the classical self–non-self model; however, it cannot explain immune responses to transplants or tumors (where no pattern recognition receptors can be involved). Nor does it cover immune responses to many viruses (which are often not evolutionarily distant) or most forms of autoimmunity.

The 'danger' model suggests that immunity might be guided by even more ancient signals, sent by damaged and dying cells, such as those involved in blood clotting⁹, or the injury-elicited signals that can lead to the activation of the NF- κ B homolog³⁷ and production of antimicrobial peptides in flies³⁸, as well as the limitation of damage and infection in plants³⁹. From this viewpoint, transplant rejection would be initiated by the surgical damage⁴⁰, viral immunity would be initiated by the virally generated damage, as well as IFN- α and heat shock proteins; and the lack of strong immunity against fetuses and tumors would be explained by the fact that almost all fetuses and tumors are healthy tissues. By limiting the amount of damage, we might thus be able to reduce the incidence and/or severity of transplant rejection⁴¹. By inducing local damage at the tumor site, or by giving tumor antigens along with danger signals, we might be able to elicit tumor immunity⁴². And when such danger signals become further characterized, we might be able to create immunological

adjuvants that mimic them to substitute for those adjuvants that, instead, generate enough damage to elicit them. Cellular danger signals might also be essential in some forms of autoimmunity, in which infection-induced damage, or mutations in any of the genes governing normal cell death or the physiological clearance of cellular debris, could cause cells to die abnormally or be inefficiently scavenged, and thus alert local APCs.

Finally, this viewpoint suggests the possibility that, like morphine/endorphin receptors in mammalian brains, many pattern recognition receptors may have evolved to bind endogenous, rather than foreign ligands. For example, CD14 (ref. 6), the LPS 'pattern recognition receptor' on macrophages, binds to apoptotic cells⁴³ and, with Toll-like receptor 2, binds LPS and bacterial lipoproteins⁴⁴, but the consequences of binding are not the same. Binding to apoptotic cells leads the macrophages to phagocytose and clear the apoptotic debris⁴³, whereas binding to LPS leads to death of the macrophages themselves⁴⁵. Perhaps the pattern recognition receptors, like many other cell surface molecules that

have been co-opted by pathogens for their own uses^{46–49}, are the quarry of, rather than the sentinels against, the microbial products they bind.

An immune system tuned to internal signals has the ability to add context to the plethora of external signals with which it is bombarded. It is able to continuously reestablish self-tolerance during its lifetime, to discriminate between harmful and innocuous foreign substances, and to generate immune responses only when they are necessary. Viewed from this vantage point, a host of new experimental and therapeutic approaches might begin to emerge.

Methods

DC cultures. Bone marrow cells from male or female B10.SCID (severe combined immunodeficiency) mice, bred at the National Institutes of Health, or C57BL/6 recombination activation gene (Rag) knockout mice (B6 RAG KO; Taconic Farms, New York) were cultured for 5–8 days at a concentration of 1×10^6 cells per well in 1 ml Iscove's modified Dulbecco's medium plus 10% FCS, glutamine, β -mercaptoethanol, antibiotics, 1–3 ng/ml granulocyte–monocyte colony-stimulating factor (PharMingen, San Diego, California) and 5 ng/ml IL-4 (BioSource, Camarillo, California); half the medium was replaced every other day. Recovery averaged 0.5×10^6 cells per well. Because of variability in the levels of constitutive activation as well as in stimulation, each experiment had its own positive (LPS) and negative (untreated) controls, and results were compared only within and not between experiments. The use of immunodeficient mice allowed us to culture fresh bone marrow cells, without depleting them of the usual T- and B-cell contaminants. Dendritic cells arising in these undepleted cultures behave identically to those from normal mice, and the yield per mouse is higher.

FACS staining. DCs were collected into cold PBS plus 1% dialyzed bovine serum albumin and 5 mM EDTA, then washed and stained on ice for 10 min with purified 2.4G2 (rat IgG2b, antibody against Fc γ RII), followed by vari-

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ous combinations of the following monoclonal antibodies: rat antibody against mouse B7.2 (GL1, IgG2a) or Ia (M5/114) (Boehringer); mouse antibody against mouse H-2D^b (KH95, IgG2b) and/or H-2D^d (34-2-12, IgG2a); hamster antibody against mouse CD40 (HM40-3, IgM), B7.1 (16-10A1, IgG) and CD11c (HL3, IgG) (PharMingen, San Diego, California). All antibodies were phycoerythrin-conjugated except antibodies against CD40 and H-2D^b, which were fluorescein isothiocyanate-conjugated, and antibody against CD11c, which was biotinylated and followed by streptavidin-red670 (PharMingen, San Diego, California).

Fibroblasts. Adult femoral quadriceps of B10.SCID and BALB/c mice were minced and plated in 10% FCS in Iscove's modified Dulbecco's medium without added growth factors. Debris were removed after 1 week and the adherent cells trypsinized and expanded. By day 15, only fibroblasts were growing. We were able to sub-culture these fibroblast preparations for more than 30 doublings without seeing any transformation.

Damaged vessels. Femoral veins were stripped from a BALB/c mouse immediately after death, mashed through a nylon filter in 400 μ l PBS, and centrifuged at 2,000g to remove any remaining viable cells and large cellular debris.

Stimulation *in vitro*. Fibroblasts (1×10^6) were added to the DCs in 50 μ l medium per 1-ml well. For living cells, fibroblasts growing in log phase were detached using 5 mM EDTA, and were washed once, resuspended in medium, and added to the DC cultures. Necrotic cells were detached, washed, and frozen and thawed four to five times. For apoptotic cells, cells were incubated for 6–8 or 12 hours in 100 μ M ceramide (Biomol, Plymouth Meeting, Pennsylvania), with 50 μ g/ml mitomycin c for 30 min at 37 °C followed by overnight incubation in serum-free conditions, then washed several times to eliminate residual apoptotic agents. We checked apoptosis efficiency by staining with the phosphatidylserine-binding protein annexin-V (PharMingen, San Diego, California) and propidium iodide exclusion (Fluka, Buchs, Switzerland). We also titrated the ceramide directly onto the DCs to determine the dose at which DCs respond. This dose (50 μ M) is far higher than the residue left after apoptotic cells were washed. Lipopolysaccharide (*Escherichia coli* 026:B6) was from Sigma and recombinant IFN- α (activity, 1×10^9 units/mg) was from Life Technologies.

T-cell proliferation assay. For the stimulation of the H-Y-specific, Th1 CD4 T-cell clone Marilyn, DCs were incubated for 40 h with necrotic or apoptotic fibroblasts, washed, irradiated (1,500 rad) and titrated into 0.2-ml wells with 5×10^4 Marilyn cells per well. After 56 h, ³H-thymidine was added and the amount of incorporation was assessed at 72 h. The DCs were irradiated to prevent some ³H-thymidine incorporation. The fibroblasts were treated with mitomycin C before being added to the DC cultures because we found that irradiation does not stop their proliferation. Preliminary experiments showed that mitomycin C did not effect their ability to stimulate DC activation. T-cell proliferation assays have the advantage of measuring the stimulatory capacity of the DCs, but the disadvantage is that they take 3 days. This allows for a small amount of stimulation on the second day by DCs that were activated by the manipulations necessary to prepare them for the assay. However, as most of the measured proliferation is induced in the first day, the short-term co-culture of DCs with T cells lessens the effect of this late activation.

Phagocytosis. BALB/c fibroblasts were stained for 10 min at 37 °C with 10 μ M carboxyfluorescein diacetate succinimidyl diester (CFSE; Molecular Probes, Eugene, Oregon) in PBS, washed twice, killed either necrotically or apoptotically, and added to the DC cultures as described above. After 24 h, the cultures were stained for CD11c and H-2D^d and analyzed by FACS (Becton Dickinson, Franklin Lakes, New Jersey).

Induction of delayed-type hypersensitivity. To sensitize the mice, we injected 10 μ l PBS, containing 10 μ g OVA mixed with stimuli, subcutaneously at the base of the tail into BALB/c mice ($n = 108$). Then, 7–8 days later, we challenged the mice by injecting 10 μ g OVA in PBS intradermally into the left hind foot and, 48 h later, measured the diameter of the foot as an assay of delayed-type hypersensitivity. Because the feet were different sizes due to age and sex differences (though the mice were always age- and sex-

matched in each experiment), we subtracted the size of the unchallenged foot from that of the challenged foot. As negative controls we used unimmunized mice. Because we had previously seen variability between cages of mice, we distributed the treated mice such that each cage contained mice in all of the treatment groups. Statistical analysis (Student's *t*-test) was used to evaluate the significance of the results.

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- Steinman, R. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9**, 271–296 (1991).
- Cella, M., Sallusto F, Lanzavecchia A. Origin, maturation and antigen presenting function of dendritic cells. *Curr. Opin. Immunol.* **9**, 10–16 (1997).
- Banchereau, J. & Steinman, R.M. Dendritic cells and the control of immunity. *Nature* **392**, 245–252 (1998).
- Pierre, P. *et al.* Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature* **388**, 787–792 (1997).
- Janeway, C.J. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* **54**, 1–13 (1989).
- Medzhitov, R. & Janeway, C.A. Jr. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* **91**, 295–298 (1997).
- Roake, J. *et al.* Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J. Exp. Med.* **181**, 2237–2247 (1995).
- Sallusto, F., Cella, M., Danieli, C. & Lanzavecchia, A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J. Exp. Med.* **182**, 389–400 (1995).
- Slupsky, P. *et al.* Activated platelets induce tissue factor expression on human umbilical vein endothelial cells by ligation of CD40. *Thromb. Haemost.* **80**, 1008–1014 (1998).
- Matzinger, P. Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* **12**, 991–1045 (1994).
- Matzinger, P. An innate sense of danger. *Semin. Immunol.* **10**, 399–415 (1998).
- Sallusto, F. & Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α . *J. Exp. Med.* **179**, 1109–1118 (1994).
- Inaba, K. *et al.* Generation of large numbers of dendritic cells from mouse bone marrow culture supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **176**, 1693–1702 (1992).
- Inaba, K. *et al.* The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells *in situ* and during maturation *in vitro*. *J. Exp. Med.* **180**, 1849–1860 (1994).
- Yahraus, T., Chandra, S., Legendre, L. & Low, P.S. Evidence for a mechanically induced oxidative burst. *Plant Physiol.* **109**, 1259–1266 (1995).
- Voll, R. *et al.* Immunosuppressive effects of apoptotic cells. *Nature* **390**, 350–351 (1997).
- Fadok, V. *et al.* Macrophages that have ingested apoptotic cells *in vitro* inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF- β , PGE₂, and PAF. *J. Clin. Invest.* **101**, 890–898 (1998).
- Rubartelli, A., Poggi, A. & Zocchi, M.R. The selective engulfment of apoptotic bodies by dendritic cells is mediated by the α (v) β 3 integrin and requires intracellular and extracellular calcium. *Eur. J. Immunol.* **27**, 1893–1900 (1997).
- Albert, M., Sauter, B. & Bhardwaj, N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392**, 86–89 (1998).
- Inaba, K. *et al.* Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J. Exp. Med.* **188**, 2163–2173 (1998).
- Kurts, C. *et al.* Constitutive class I-restricted exogenous presentation of self antigens *in vivo*. *J. Exp. Med.* **184**, 923–930 (1996).
- Germain, R. & Margulies, D.H. The biochemistry and cell biology of antigen processing and presentation. *Annu. Rev. Immunol.* **11**, 403–450 (1993).
- Bradley, J. Indirect T cell recognition in allograft rejection. *Int. Rev. Immunol.* **13**, 245–255 (1996).
- Sen, G. & Lengye, I.P. The interferon system. A bird's eye view of its biochemistry. *J. Biol. Chem.* **267**, 5017–5020 (1992).
- Pfeffer, L. *et al.* Biological properties of recombinant alpha-interferons: 40th an-

- niversary of the discovery of interferons. *Cancer Res.* **58**, 2489–2499 (1998).
26. Swensson, H., Johannisson, A., Nikkila, T., Alm, G.V. & Cederblad, B. The cell surface phenotype of human interferon-alpha producing cells as determined by flow cytometry. *Scand. J. Immunol.* **44**, 164–172 (1996).
27. Chakrabarti, D., Hultgren, B. & Stewart, T.A. IFN-alpha induces autoimmune T cells through the induction of intracellular adhesion molecule-1 and B7.2. *J. Immunol.* **157**, 522–528 (1996).
28. Luft, T. *et al.* Type I IFNs enhance the terminal differentiation of dendritic cells. *J. Immunol.* **161**, 1947–1953 (1998).
29. Gresser, I., Tovey, M.G., Bandu, M.E., Maury, C. & Brouty-Boye, D. Role of interferon in the pathogenesis of virus diseases in mice as demonstrated by the use of anti-interferon serum. I. Rapid evolution of encephalomyocarditis virus infection. *J. Exp. Med.* **144**, 1305–1315 (1976).
30. Lassila, O., Vainio, O. & Matzinger, P. Can B cells turn on virgin T cells? *Nature* **334**, 253–255 (1988).
31. Ochsenbein, A.F. *et al.* Immune surveillance against a solid tumor fails because of immunological ignorance. *Proc Natl. Acad. Sci. USA* **96**, 2233–2238 (1999).
32. Ronchetti, A. *et al.* Immunogenicity of apoptotic cells *in vivo*: role of antigen load, antigen-presenting cells, and cytokines. *J. Immunol.* **163**, 130–136 (1999).
33. Harding, C., Heuser, J. & Stahl, P. Endocytosis and intracellular processing of transferrin and colloidal gold-transferrin in rat reticulocytes: demonstration of a pathway for receptor shedding. *Eur. J. Cell Biol.* **35**, 256–263 (1984).
34. Bartocioni, E. *et al.* MHC class I, MHC class II and intercellular adhesion molecule-1 (ICAM-1) expression in inflammatory myopathies. *Clin. Exp. Immunol.* **95**, 166–172 (1994).
35. Mackay, C., Marston, W.L. & Dudler, L. Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J. Exp. Med.* **171**, 801–817 (1990).
36. Tamura, Y., Peng, P., Liu, K., Daou, M. & Srivastava, P.K. Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science* **278**, 117–120 (1997).
37. Ip, Y. *et al.* Dif, a dorsal-related gene that mediates an immune response in *Drosophila*. *Cell* **75**, 753–763 (1993).
38. Lemaitre, B., Reichhart, J.M. & Hoffmann, J.A. *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proc. Natl. Acad. Sci. USA* **94**, 14614–14619 (1997).
39. Delledonne, M., Xia, Y., Dixon, R.A. & Lamb, C. Nitric oxide functions as a signal in plant disease resistance. *Nature* **394**, 585–588 (1998).
40. Cole, W. Efforts to explain spontaneous regression of cancer. *J. Surg. Oncol.* **17**, 201–209 (1981).
41. Land, W. *et al.* The beneficial effect of human recombinant superoxide dismutase on acute and chronic rejection events in recipients of cadaveric renal transplants. *Transplantation* **57** (1994).
42. Coley, W. The treatment of inoperable sarcoma with the mixed toxins of erysipelas and bacillus prodigiosus. *JAMA* **20**, 389–395 (1898).
43. Devitt, A. *et al.* Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature* **392**, 505–509 (1998).
44. Yang, R. *et al.* Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* **395**, 284–288 (1998).
45. Aliprantis, A. *et al.* Cell Activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2. *Science* **285**, 736–739 (1999).
46. Cohen, O., Kinter, A. & Fauci, A.S. Host factors in the pathogenesis of HIV disease. *Immunol. Rev.* **159**, 31–48 (1997).
47. Fingerhuth, J. *et al.* Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. *Proc. Natl. Acad. Sci. USA* **81**, 4510–4514 (1984).
48. White, J. *et al.* The V beta-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell* **56**, 27–35 (1989).
49. Joiner, K., Brown E.J. & Frank, M.M. Complement and bacteria: chemistry and biology in host defense. *Annu. Rev. Immunol.* **2**, 461–491 (1984).