OPINION

Death-defying immunity: do apoptotic cells influence antigen processing and presentation?

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The clearance of apoptotic cells has been paid much attention for its role not only in tissue homeostasis, but also as a source of antigen for immune tolerance and activation. The complexity of this process has been borne out by the many receptor families and signalling pathways involved; however, an important aspect of the biology has so far been overlooked. This article explores the possible immunological instructions that are delivered by dying cells, as influenced by the specific execution pathways that are active during programmed cell death.

Multicellular organisms have evolved genetic and epigenetic mechanisms of programmed cell death for the elimination of cells that are no longer required or have become damaged. Phagocytes engulf the dying cells, in turn maintaining tissue homeostasis. Recent evidence indicates that dying cells have evolved mechanisms to transfer immunologically relevant information to their 'captor', and in doing so offer a means of signalling the nature of cell death (normal cell turnover versus reactive) and directing the immunological outcome of phagocytosis. Here, I provide an overview of the ways in which cells die, their complex relationship with tissue phagocytes and the little that is known regarding the outcome of these events. Attention will be given to dendritic cells (DCs) as they are unique among phagocytes in being capable of presenting antigenic peptides derived from internalized dying cells on MHC molecules for the activation or tolerization of T cells. Then, I propose a model for how apoptotic cells instruct DCs in the integration of the information they gather.

The signs and signifiers of cell death

In the late nineteenth century, Rudolph Virchow characterized the morphological features of cell death[1]. Necrosis was defined by localized death of living tissue as noted by the change in colour and by evidence of inflammation. On a cellular level, Virchow noted cell swelling and the loss of membrane integrity. Today, necrosis (or primary necrosis) is defined as passive death, triggered by noxious stimuli, including extreme temperatures, toxins and hypotonic conditions[2,3] (BOX 1). The use of 'primary' to describe necrosis is to distinguish it as being the mechanism of death. By contrast, secondary necrosis denotes a cell that died by apoptosis, but due to a defect in clearance (see later) has lost membrane integrity. Here, I use necrosis to describe what others refer to as primary necrosis, unless otherwise stated. Such triggers of necrotic death affect large numbers of cells in a tissue micro-environment and result in the diffusion of cellular contents, which in turn provokes the release of inflammatory signals from surrounding, living cells[4]. Importantly, necrotic cell death is not under genetic control, and death by necrosis does not result in the use of energy (for example, ATP). In fact, these two criteria constitute the operational definition of necrosis that is used here.

The term apoptosis was first used to describe controlled cell deletion by Kerr, Wylie and Currie in 1972 (REFS 5,6). It was characterized morphologically on the basis of nuclear and cytoplasmic condensations and the breaking up of the cell into numerous membrane-bound vesicles, known as apoptotic bodies. In the later stages of apoptosis, it was noted that the cell bodies were shed or internalized by phagocytes. Based on these microscopic observations, it was concluded that apoptosis (later named programmed cell death) is an active phenomenon that is involved in cell turnover as a balance to mitosis (BOX 1).

To these morphological criteria have now been added biochemical features of programmed cell death, which include exposure of phosphatidylserine on the outer leaflet of the plasma membrane, proteolytic cleavage of many intracellular molecules (for example, poly-ADP-ribose polymerase, PARP) and the internucleosomal cleavage of DNA, resulting in the observed DNA laddering in late-stage apoptotic cells[7–9]. Although these specific morphological and biochemical changes can be used as guidelines for monitoring a cell in the process of dying, it is important to consider that there are many molecular pathways that result in death — and with a spectrum of death programmes, there exists the intriguing possibility that distinct immunological responses might result from the different mechanisms by which cells die.

As I discuss, the central point is that in distinguishing cell-death pathways we might uncover important mechanisms by which dying cells control immune responsiveness. In wild-type cells, a complex integration of cell-death pathways occurs[10], but I argue that the triggering of distinct cell-death pathways results in the generation of bioactive agents that influence immunity as measured by the effect of the dying cell on phagocytes and, in turn, the adaptive immune system.

Box 1 | A molecular definition for death

Necrotic cell death

Although there has been little attention given to the passive release of intracellular molecules that are secondary to necrotic death, there are a few important molecules to consider. High mobility group box 1 (HMGB1), a chromatin-binding protein, is passively released during necrosis, and outside the cell, it binds the receptor for advanced glycosylation end products (RAGE)[66,67]. It functions as a diffusible signal of unprogrammed cell death, triggering a nuclear factor-κB (NF-κB) signalling pathway in neighbouring cells[68]. By contrast, apoptotic cells do not release HMGB1 (REF. 67). This is a result of active de-acetylation of histones, therefore resulting in tight binding of HMGB1 to chromatin. Of note, no release of HMGB1 was evident even after partial autolysis ensued. More recently, Shi *et al.*[69] showed that uric acid serves as another diffusible danger signal; however, it remains to be shown how apoptotic cells (or the phagocyte) quench this inflammatory agent.

Heat-shock proteins (HSPs) are another source of inflammatory cues and act as chaperones of antigenic peptides[40]. *In vivo* mouse experiments indicate that adoptively transferred peptide–HSP complexes from tumour cells or virus-infected cells elicit protective immunity that is restricted to cognate antigen[70,71]. Dying cells release HSPs that are in turn captured by cell-surface receptors on antigen-presenting cells, thereby facilitating antigen transfer and presentation by MHC molecules. In addition, there is now evidence that HSPs are pro-inflammatory, based on the release of tumour-necrosis factor (TNF) and interleukin-1β (IL-1β), and the induction of dendritic-cell (DC) maturation[40,72].

Apoptotic cell death

Ellis and Horvitz[73] first discovered the molecular nature of programmed cell death involving cell death abnormal (*ced*) genes in *Caenorhabditis elegans*. When *ced-3* was cloned, the gene product revealed a marked homology to the mouse and human protein IL-1β-converting enzyme (ICE), also known as caspase-1 (REF. 74).

Recent studies, however, indicate that caspases are not essential for cell death in mammalian cells[75]. For example, the generation of intracellular reactive oxygen species (ROS) that are secondary to death-receptor ligation results in mitochondrial membrane permeability[76]. Whereas some have called these caspase-independent pathways 'sub-apoptosis' or even necrosis owing to the absence of the classical morphological features of apoptosis, these death pathways are energy dependent, and *in vivo*, phagocytes can capture cells without release of their intracellular contents. Although the biochemical details of these death pathways are beyond the scope of this Opinion article, the important point is that distinct pathways exist for death and removal of dead cells. These may be distinguished by the apoptotic trigger, the cell type undergoing programmed cell death and the phagocyte responsible for clearance.

Dying cells modulate innate immunity

Until recently, little was known about the molecular mechanisms responsible for the ligation and internalization of apoptotic cells by phagocytes, but in the past few years there have been several molecules implicated in this process. Importantly, it is believed that all cells (with the exception of lymphocytes and red blood cells) are capable of phagocytosis. The ability to internalize dying cells has been shown in a wide variety of cell types, including so-called 'non-professional' phagocytes such as mesangial cells and retinal pigmented-epithelial cells, and of course the 'professional' phagocytes, such as macrophages and DCs. What is pertinent for this discussion is an understanding that the immunological outcome of cell death is determined indirectly as measured by the effect on neighbouring cells, the phagocytes that internalize the dying cell (FIG. 1), or even more distally (for example, in the draining lymph node) by the interaction of these phagocytes with cells of the adaptive immune system (see later).

Skewing cytokine production by phagocytes. Necrotic death was originally defined as inflammatory death (BOX 1) and apoptosis as an inert event ending in degradation by a phagocyte; for nearly 25, years the internalization of apoptotic cells was considered to have no immunological role. However, data now indicate that apoptotic cells are not simply degraded. Instead, they have an active role in modulating both innate and adaptive immunity. For example, macrophages efficiently engulf apoptotic cells and the process of phagocytosis actively inhibits the production of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), IL-12 and tumour-necrosis factor (TNF), in response to lipopolysaccharide (LPS) challenge[11,12]. In turn, the production of transforming growth factor-β1 (TGF-β1) and platelet-activating factor (PAF) is increased — both are considered to be immunosuppressive, as addition of exogenous TGF-β1 or PAF results in the inhibition of Toll-like receptor 4 (TLR4)-mediated cytokine production[13]. Interestingly, follow-up studies indicate that use of receptors by macrophages for the binding and internalization of apoptotic cells is crucial. Internalization of latex beads is immunologically silent; crosslinking CD36 (a co-receptor for integrin $\alpha_v\beta_3$) or the phosphatidylserine receptor on macrophages mimics the anti-inflammatory effect of apoptotic cells, and the phagocytosis of antibody-opsonized apoptotic cells is pro-inflammatory[7,14,15]. Similar data are now available for DCs[16]. The distinct outcomes observed are in part dependent on the opsonin available for coating the dying cell and the microenvironment in which the apoptotic cell dies (FIG. 1). One important caveat is that the monitoring of immune suppression depends on challenge with an inflammatory agent (for example, LPS); in other words, what is being characterized is the role apoptotic cells have in helping the tissue return to homeostasis (this should be distinguished from maintaining homeostasis). In fact, there are few data available on the effects of normal cell turnover, situations in which the phagocytes are likely to be neighbouring cells, not professional phagocytes. The consequences of inefficient clearance of dying cells has particular relevance to the induction and maintenance of systemic autoimmunity; this has been reviewed elsewhere[3] and is not addressed in detail here.

Secretion of bio-active lipids and phagocyte recruitment. As dying cells are for most researchers considered to be an endpoint, not much is known about the cytokines or chemokines they secrete during death; the nucleic acid and protein they package for transfer to the phagocytes that internalize them; or the carbohydrate modifications that occur as a result of programmed cell death. One emerging field, however, is the study of bio-active lipids that are actively generated as a result of caspase activation during programmed cell death (FIG. 1). This work has been pioneered by Richard Kolesnick and others[17,18], who showed that ligation of death receptors results in early activation of an acid-pH-dependent sphingomyelinase, which catabolizes sphingomyelin present in the outer leaflet of the plasma membrane, resulting in the generation of ceramide and in turn sphingosine-1-phosphate (S1P). Much attention has been given to the role ceramide and S1P have intracellularly in facilitating cell death and survival, respectively. But slowly the field is opening up to the idea that both ceramide and S1P are released by dying cells, in turn influencing the micro-environment of death. With respect to S1P, its role as an extracellular ligand was first

appreciated when it was shown to bind and signal through the orphan receptor endothelial-derived G-protein-coupled receptor 1 (EDG1)[19,20]. Further insight into the biology emerged with the identification of the immunomodulatory sphingosine analogues FTY720 and its phosphoryl metabolite, which affect the trafficking of lymphocytes[21].

More recently, a second lysophospholipid that is associated with apoptosis has been implicated in cell migration. Lauber *et al.*[22] showed that caspase-3 activation results in phospholipase A_2 (PLA_2)-mediated processing of phosphatidylcholine into lysophosphatidylcholine (LPC) and arachadonic acid,

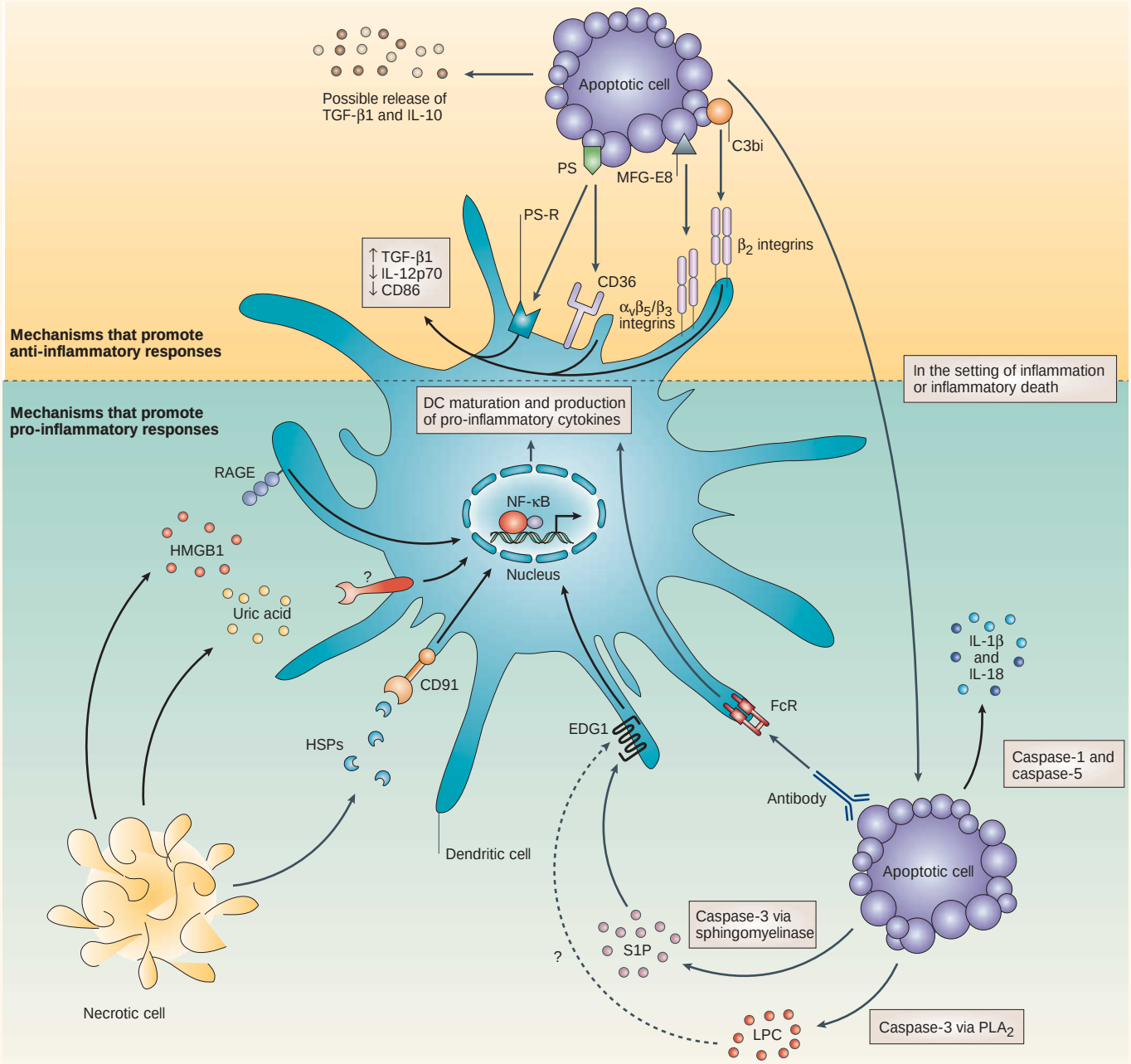

Figure 1 | **Indirect and direct effects of dying cells on the phagocytes that engulf them.** The immunological outcome of cell death is determined by both indirect and direct effects on phagocytes. Anti-inflammatory responses might be triggered as a result of particular receptor usage. It has been shown in dendritic cells (DCs) that ligation of CD36, phosphatidylserine (PS) receptor and β_2 integrins (all receptors for apoptotic cells) results in the increased release of transforming growth factor-β1 (TGF-β1), and the downregulation of interleukin-12 (IL-12) expression, as well as some markers of DC activation (such as CD86). Reports also indicate that some apoptotic cells themselves are a potential source of IL-10 and TGF-β1. If antibodies that can bind and opsonize apoptotic cells are present (such as PS-specific antibodies, which are present in a subset of patients with systemic lupus erythematosus), ligation of Fc receptors (FcRs) will result in DC maturation and the production of immunostimulatory cytokines. Inflammation might also occur secondary to apoptotic death pathways that activate caspase-1 or result in the release of sphingosine-1-phosphate (S1P) and lysophosphatidylcholine (LPC), which signal through endothelial-derived G-protein-coupled (EDG) receptors. In the event of necrosis, passive release of high mobility group box 1 (HMGB1), uric acid and heat-shock proteins (HSPs) all have a potential role in stimulating inflammation through a nuclear factor-κB (NF-κB)-mediated pathway. These latter inflammatory stimuli may also be important in certain types of apoptotic cell death, in which case signalling probably occurs from within the phagosome — it remains to be determined whether inflammatory death pathways result in decreased acetylase activity, therefore allowing release of HMGB1, or in enhanced formation of uric acid crystals. PLA_2, phospholipase A_2; RAGE, receptor for advanced glycation end products.

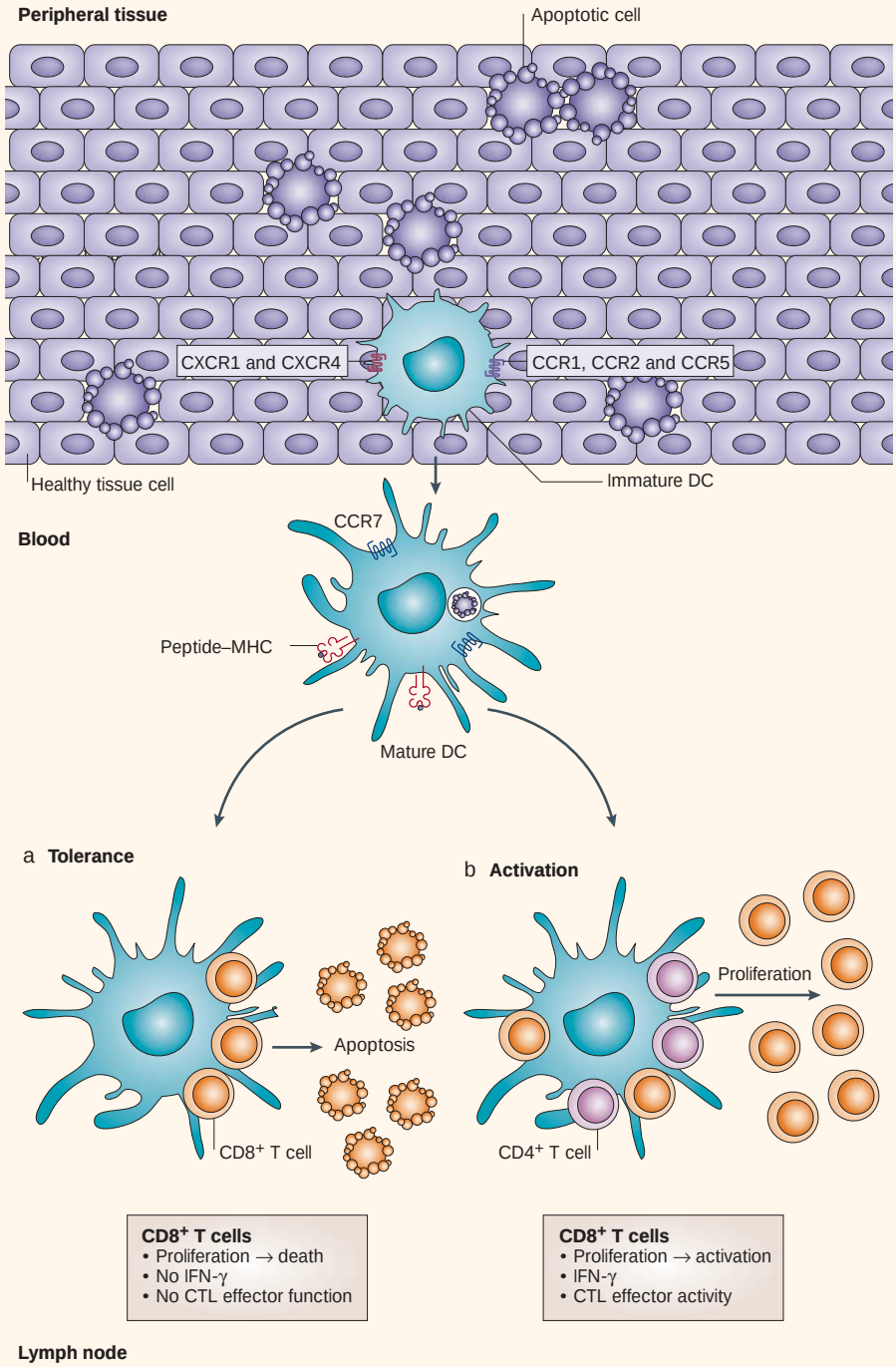

Figure 2 | **Apoptotic cells captured in the periphery by dendritic cells serve as a source of antigen for the tolerization and activation of CD8⁺ T-cell responses.** The proposed *in vivo* pathways by which immature dendritic cells (DCs) capture antigen from dying cells in the periphery and induce tolerance (**a**) or the activation (**b**) of CD8⁺ T cells. **a** | The tolerogenic response depends on the microenvironment of the lymph node, the absence of antigen-specific CD4⁺ T-cell help or death through anti-inflammatory, homeostatic pathways. Tolerance of CD8⁺ T cells occurs through a deletional mechanism, whereby antigen-reactive cells divide and die in the absence of interferon-γ (IFN-γ) production or the generation of effector cytotoxic T lymphocytes (CTLs). **b** | By contrast, the ability to activate CD8⁺ T cells depends on the presence of stimuli capable of activating DCs or antigen-specific CD4⁺ T-cell help, or might occur as a result of inflammatory death (for example, apoptosis involving the activation of caspase-1/caspase-5). Once activated, these CD8⁺ T cells return to the site of inflammation and lyse target cells (for example, virus-infected cells or cells on their way to malignant transformation). One important consideration is that for either tolerance or activation, cell-surface expression of CC-chemokine receptor 7 (CCR7) must occur, thereby facilitating the trafficking of DCs to the lymph node for interaction with T cells. CXCR, CXC-chemokine receptor.

both of which are released by apoptotic cells. Interestingly, LPC acts as a chemoattractant for monocytes and macrophages. Although single apoptotic cells are probably internalized by neighbouring cells or resident tissue phagocytes, it is interesting to consider that large numbers of dying cells could actively participate in their own removal by cell recruitment[22]. Alternatively, the release of LPC could be considered as pro-inflammatory — macrophage infiltration is in fact a hallmark of inflammation. This classification would be supported if it turns out that, similar to S1P, LPC signals through EDG receptors, which are known to signal through nuclear factor-κB (NF-κB). Clearly, more work is required to define the effects of different death stimuli on the generation of bio-active lipids, thereby allowing classification of these modulating agents as promoting inflammation versus facilitating the maintenance of homeostasis.

Dying cells modulate adaptive immunity

Apoptotic cells are a source of antigen for dendritic cells. In addition to their indirect effects on innate immunity, apoptotic cells have been shown to have an important role in adaptive immune responses — they serve as a source of antigen. Our *in vitro* studies and the more recent *in vivo* work of others show that immature DCs can capture apoptotic cells and cross-present antigen derived from internalized dying cells on MHC class I molecules for recognition by CD8⁺ T cells[23–27] (FIG. 2). DC trafficking of antigen derived from internalized apoptotic cells has been shown for models of gut-, skin- and pancreas-restricted antigen[28–31]. In the last model system, the use of transgenic mice that express inhibitors of apoptosis in islet β-cells and the *in vivo* injection of biochemical modulators of cell-death pathways have both confirmed the important role for cell death in antigen transfer and T-cell activation/tolerance[26,30]. To appreciate the efficiency of antigen transfer in this exogenous pathway, it is important to consider the characteristic features of apoptotic cells. In all cases of programmed cell death, cell-membrane integrity is maintained. In addition, the dying cell exposes novel and altered surface markers on its plasma membrane, thereby facilitating recognition and rapid internalization[3]. So, when serving as the phagocyte, DCs probably capture an entire dying cell and with it, its full repertoire of antigenic information.

One means of antigen transfer would be the trafficking of whole protein from the apoptotic cell to the MHC class-I-presentation pathway of the DC through a phagosome-to-cytosol pathway[32]. Recent publications from

the groups of Amigorena[32] and Desjardins[34] indicate that internalized antigen escapes from a fusion phagosome–endoplasmic reticulum (ER) compartment through Sec61, allowing antigen processing by the DC proteasome. Alternatively, the apoptotic cells might have an active role in antigen presentation through the delivery of 'pre-processed' peptide epitopes. This would provide an efficient means of generating peptide–MHC class I complexes in the DC and place the control of epitope selection with the dying cell. We have recently identified such a pathway, demonstrating that peptides from within the ER of dying cells are loaded onto MHC class I molecules in DCs without the requirement for additional cytosolic processing (N. E. Blachère, M.L.A. and R. B. Darnell, unpublished observations). Such a pathway would offer a role for the heat-shock proteins (HSPs) GP96 and calreticulin. In fact, we favour this scenario as the main pathway by which HSPs transfer antigen (FIG. 3). Indeed, the report of phagosome–ER fusion[35] would allow peptide–HSP complexes to interact directly with MHC class I molecules of the DC. As ER chaperones in the phagocytosed cell would be bound to the pool of peptides derived from newly synthesized proteins, they offer the DC an accurate representation of what occurred immediately before death; this might be particularly relevant in the setting of an acute infection that triggers apoptotic cell death.

Outcomes of cross-presentation

Apoptosis versus necrosis. Our initial studies demonstrated a crucial requirement for apoptotic cell death to achieve antigen cross-presentation, and although these results have now been confirmed by others, there continues to be some controversy in the literature. In one of the earliest examples, Melcher *et al.*[36] described an *in vivo* system in which the killing of tumour cells by 'non-apoptotic pathways' is associated with high immunogenicity of the expressed tumour antigens. However, it is confusing that Melcher *et al.* consider the cell death that was studied to be necrotic as they report upregulation of HSP expression. In light of this consideration, their studies might in fact be consistent with the pathway we described; however, classification of the cell death responsible for the transfer of antigen to antigen-presenting cells (APCs) requires clarification.

A more direct challenge comes from the work of Larsson and colleagues[37,38], who show in two viral models that apoptotic and necrotic material are equally potent in charging DCs with antigen for the loading of MHC class I molecules. Larsson uses infection with vaccinia virus (by their own accounts triggering marked apoptosis in infected cells) or cell lines that are known to die by apoptosis as a result of infection with influenza virus. Accordingly, their studies are comparing apoptosis with secondary necrosis (achieved by freeze–thaw 4–8 hours into a programmed cell-death pathway). As further evidence for an active process being involved in their so-called necrotic death, similar to Melcher, they report upregulation of HSP expression and argue that peptide–HSP complexes in their lysates are mediating antigen transfer. Although we may be entering semantic issues concerning cell death, it is important to be rigorous in our definitions. In support of this re-interpretation, it is interesting to consider the *in vivo* comparison of apoptosis versus necrosis in tumour models — here, the antigen does not need to be introduced and the cells killed by necrosis have not had their death effector pathways triggered. In these studies, the results show a clear role for apoptotic cells in allowing efficient antigen delivery for cross-priming and tumour protection[39].

One important point that this debate has revealed is the importance of inducible peptide–HSP complexes in the delivery of antigen to the immune system, in particular to DCs, for the activation of $CD8^+$ T cells. These insights led to a refined model in which necrotic death allows passive release of HSPs that are inflammatory and when captured by cell-surface receptors on DCs (for example, CD91), might result in cross-priming of cytotoxic T lymphocytes (CTLs)[40,41]. Again I suggest an alternative interpretation with respect to the role of HSPs in physiological and pathological situations. Notably, experiments with whole protein show that it is possible to achieve cross-presentation by increasing antigen concentration[42]. It is therefore no surprise that cell lysates or purified peptide–HSP complexes can prime CTLs. But given the fact that α2-macroglobulin (α2-M), which is the second most abundant plasma protein, is a competitive ligand for HSPs in the binding of CD91 (REF. 43), I am unsure whether *in situ* release of HSPs can compete. A counter argument might be made: given the large numbers of cells that would die in response to a necrotic stimulus, there would be release of high concentrations of HSPs that might in fact compete with α2-M; however, the necrotic stimulus would have also killed the APCs in the region suggesting that infiltrating macrophages (not DCs) would be the dominant phagocyte. Furthermore, as eluded to earlier, a true necrotic stimulus would not allow upregulation of expression of inducible HSPs.

One testable hypothesis that will help to redefine the existing model concerns our proposed role for HSPs as the active carrier of peptide antigen in apoptotic cells (FIG. 3). In such a scenario, the peptide–HSP complexes would not have to compete with α2-M at the cell surface. Loading of MHC class I molecules might occur in the phagolysosome after fusion with the ER[35]; alternatively, CD91 might be responsible for shuttling HSPs from the endosome to the trans-Golgi network and then to the ER[44–46]. Our observation that lactacystin treatment of DCs only partially inhibited cross-presentation indicated that a proteasome-independent pathway exists for the loading of MHC class I molecules[23]. Although there is probably an important role for antigen derived from apoptotic cells (be it processed by the apoptotic cell or the DC), we are still left with the question as to how the distinct outcomes of antigen cross-presentation are regulated.

Priming versus tolerance. Although the uptake of apoptotic cells by DCs results in the activation (or cross-priming) of CTLs[23], this mechanism of antigen presentation might also be important for the induction of cross-tolerance. In a series of inspired studies, Heath and colleagues[47] have shown that neo-self, tissue-restricted antigen could be captured and cross-presented by bone-marrow-derived cells. These APCs trafficked the captured antigen to the draining lymph node where interaction with antigen-specific, MHC class-I-restricted T cells resulted in their clonal expansion and active deletion (or tolerance). By contrast, when helper T cells were included in the adoptive transfer, the $CD8^+$ T-cell population expanded and differentiated into effector cells[48]. It was therefore proposed that a bone-marrow-derived cell captures exogenous antigen for both MHC class I and MHC class II presentation, transports these epitopes to the draining lymph node and presents them to naive $CD4^+$ and $CD8^+$ T cells. It has now been shown that the cross-presenting APC that is responsible for cross-tolerance is indeed the DC[49,50]. Furthermore, it is probable that apoptotic cells are responsible for transferring antigen to DCs in this and other models, as the cross-presentation of tissue-restricted antigen is enhanced when pro-apoptotic stimuli are included[51]; and the reciprocal is also true — expression of anti-apoptotic molecules (for example, CrmA) prevented efficient cross-tolerization of tissue-restricted antigen[26].

So, concerning the immunological outcome of activation versus tolerance, one important point of regulation has emerged. In the absence of antigen-specific cognate $CD4^+$ T-cell help, DCs that cross-present antigen to $CD8^+$ T cells result in tolerance by a deletional mechanism[48,50]. Although there is much effort committed to the so-called 'three-cell problem', we instead concern ourselves with a more upstream issue — that is, the possibility that cell death is another point of regulation that governs this immunological decision.

Death: upstream regulator of immunity

Homeostatic versus inflammatory apoptosis. Physiological programmed cell death has an essential role in development, differentiation and tissue homeostasis, but it has only recently been accepted that apoptotic death might also be inflammatory[52,53]. I suggest the separation of homeostatic death from inflammatory death, with the former describing programmed cell death during developmental processes (including aging of cells). This is probably a death from within and, with respect to the adult organism, may occur as a result of the accumulation of reactive oxygen species (ROS). As it is not often considered, the focus here is inflammatory death.

Caspase-1 (or IL-1β-converting enzyme, ICE) is now known to be one of four inflammatory caspases (the others are caspase-4, -5 and -13; caspase-11 and -12 are the mouse homologues)[54]. Knockout mice that lack caspase-1, -11 and -12 have been generated and all have selective defects in cell death. With respect to their role in inflammation, caspase-1 and caspase-5 have been shown to associate with PYCARD/ASC and NALP1, a pyrin domain-containing protein with homology to nucleotide-binding oligomerization domain (NOD) proteins[55]. Together, these proteins constitute what has been called the inflammasome — a large signal-induced multiprotein complex that results in the activation of pro-inflammatory caspases and the cleavage of IL-1β and IL-18 precursors (REF. 56). This link between secretion of

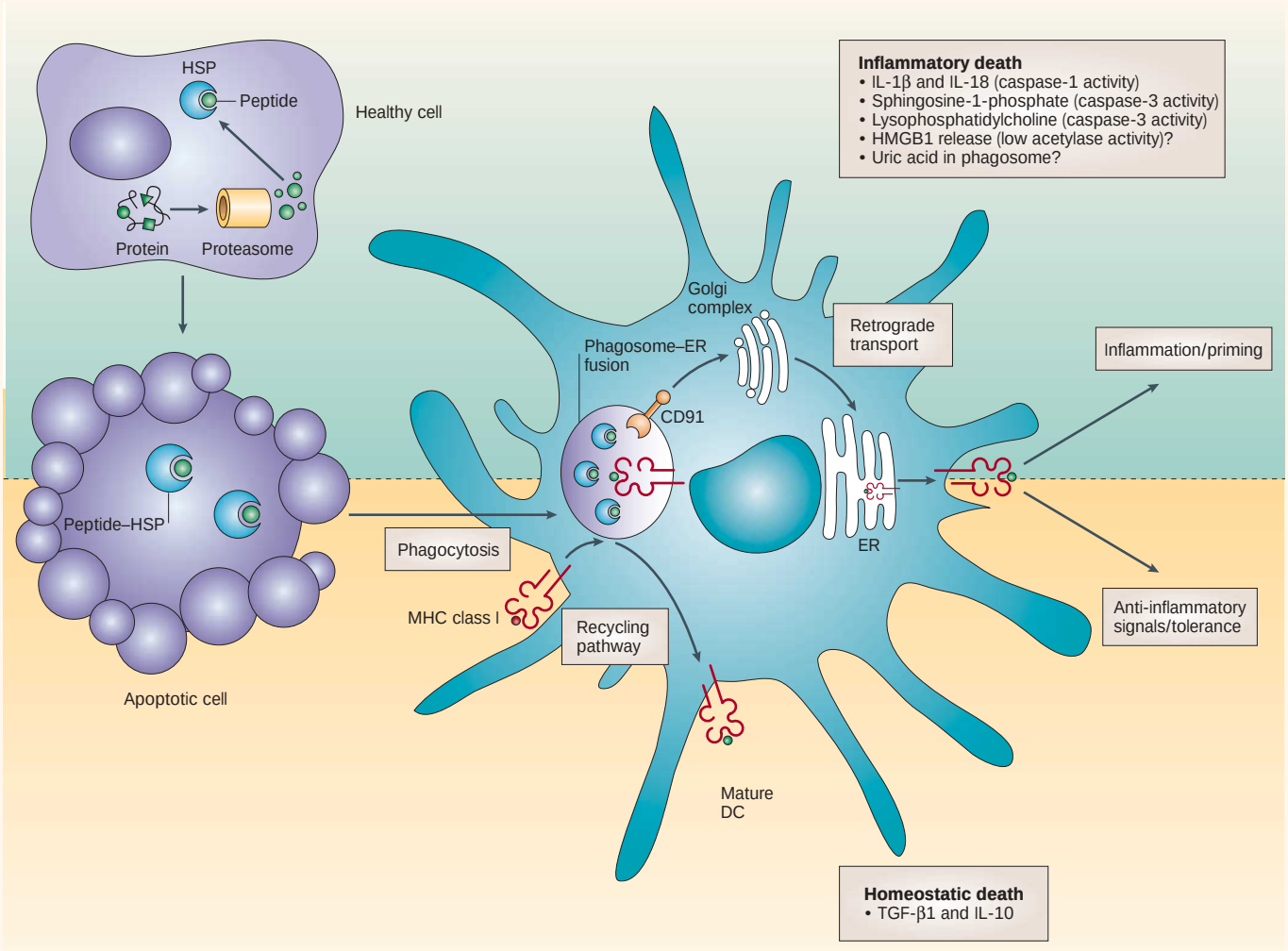

Figure 3 | **An active role for apoptotic cells in the transfer of antigen to dendritic cells.** We propose that apoptotic cells have an active, energy-dependent role in immunity, both through the transfer of 'pre-processed' antigen as well as through cytokines, lipids and carbohydrate moieties that are generated/secreted secondary to the use of specific death effector pathways. With respect to antigen transfer, such a mechanism would provide an efficient means of generating peptide–MHC class I complexes in the dendritic cell (DC), placing the control of epitope selection in the hands of the dying cell. DRiPs (defective ribosomal initiation products) chaperoned by heat-shock proteins (HSPs) are the probable source of antigen; and given the description of phagosome–endoplasmic reticulum (ER) fusion, it is possible for peptide–HSP complexes to interact directly with MHC class I molecules in DCs. Alternatively, the HSPs might traffic through a retrograde transport pathway, shuttled by CD91 to the trans-Golgi and to the ER by binding to KDEL-receptor. As ER chaperones in the phagocytosed cell would be bound to the pool of peptides derived from newly synthesized proteins, they offer the DC an accurate representation of what occurred immediately before death; this may be particularly relevant in the setting of an acute infection that triggers apoptotic cell death. Depending on the type of apoptotic cell death and/or the micro-environment of antigen capture (see FIG. 2), cross-presentation might result in tolerance or activation. HMGB1, high mobility group box 1; IL, interleukin; TGF-β1, transforming growth factor-β1.

pro-inflammatory cytokines, cell death and protective immunity must be explored. The answer will not be obvious based on the finding that caspase-1-deficient mice are resistant to LPS-induced shock[52], but are unable to resolve infection with *Shigella* (an invasive bacteria known to induce apoptotic death of professional phagocytes in the infected gut epithelium and the secretion of high levels of IL-1β and IL-18)[57].

Genetic versus epigenetic programmed cell death. Another means of delineating death pathways involves the use of inhibitors of protein synthesis or RNA translation. Whereas some triggers of programmed cell death such as FAS (CD95) ligation and γ-irradiation are blocked by cyclohexamide or actinomycin D, others have been shown to be insensitive[58]. I have termed these latter death pathways epigenetic programmed cell death, as the induction phase of apoptosis is bypassed — in other words, there is no requirement for DNA or other genetic machinery; the triggering of effector caspases is a direct reflection of the environmental conditions acting on the cell. One important example of epigenetic programmed cell death is mediated by granzyme B — a serine protease found in the cytoplasmic granules of CTLs and natural killer cells. Granzyme B accesses the cytosol of target cells through pores generated by another component of the granules, perforin[59]. Once in the cytosol, it can directly cleave pro-caspase-3, in turn activating this central effector caspase, as well as the pro-apoptotic BH3-domain only protein BID, which results in disruption of mitochondrial integrity[60,61].

From an immunological perspective, perforin/granzyme-B-mediated apoptosis is a result of immune activation. Although it is possible that such death results in feed-forward immune activation (target cells being themselves cross-presented, in turn activating additional CTLs), I fear that this would result in us becoming one large lymph node. An alternative hypothesis would be that CTL-mediated killing triggers a death effector pathway that is anti-inflammatory. This is supported by the observations of Rosen and colleagues[62,63] that most autoantigens targeted in systemic autoimmune diseases are substrates for granzyme B. It may be important to investigate polymorphisms in death effector pathways as the upstream trigger of autoimmunity. It will also be important to define how the granzyme-B-mediated death effector pathway favours a return to tissue homeostasis.

Who gets to 'eat' and how much? To understand the *in vivo* relevance of cell death and specifically the consequences of handling recently dead cells, it is essential that we consider the different micro-environments in which phagocytes 'eat' and cells die. As *in vivo* experimentation continues, I propose that we take care to distinguish the effects of apoptotic cell death in sites of low levels of programmed cell death (for example, the kidneys) from tissue with high levels of cell turnover (for example, germinal centres and the liver). Notably, the constitutive presence of macrophages in resting tissue is selective for sites of high levels of death (for example, tingible-body macrophages in the germinal centres and Kupffer cells in the liver). With respect to peripheral non-haematopoietic tissue with low levels of death, the phagocytes are typically the neighbouring cells (for example, mesangial cells in the kidney). If DCs are required for cross-presentation and communication of tissue events to the adaptive immune system, this characterization of resting tissues would suggest that, as originally proposed, most apoptotic events are immunologically inert (at least with respect to the adaptive immune system). DCs cannot compete with macrophages as they are 5–10 times less efficient at phagocytosis[23,64]. So, their opportunity for capture of apoptotic cells is a result of their presence in resting peripheral tissue. Indeed, sentinel DCs exist throughout the body and *in vivo* data indicate that although they are rare events, apoptotic-cell-laden DCs have been found in the afferent lymphatics and draining lymph nodes of many tissues[30,31]. Importantly, resting DCs exist in distinct layers of the tissue compared with macrophages (for example, in the small intestine and skin, the histology of which have been carefully analysed). During inflammation, one can envision that resident DCs get to eat early — trafficking antigen to mediate T- and B-cell activation — and that their role as the tissue phagocyte is temporally regulated. Once monocytes are recruited into the site of inflammation they dominate, having an active role in clearing the site of inflammation and restoring tissue homeostasis. All immune responses must be regulated, and the macrophage may offer the requisite negative feedback to inhibit inflammation and prepare for wound healing. In this scenario, DCs and macrophages work together to both defend infected (inflamed) tissue and clear the inflammatory site for future tissue remodelling.

Box 2 | Why death? An evolutionary perspective

Although it is provocative to consider that our highly evolved cell-death programmes are intimately linked to immunity, how might such intricate pathways have arisen in the first place? To approach such a question, it is useful to consider the evolution of apoptosis. This is thought to have occurred near the transition from single-celled to multi-cellular life, although some work indicates that the evolution of death effector pathways preceded this juncture[77]. The writings of Jean Claude Ameisen[78,79] offer creative scenarios for the evolution of programmed cell death, but do not directly predict the clearance of dying cells. Indeed, the answer to the question 'Why death?' might be phagocytosis. This was first suggested by Charles David, based on the discovery that the simple metazoan *Hydra vulgaris* survives in nutrient-deprived conditions by capturing energy from internalized apoptotic epithelial cells[80,81]. Accordingly, phagocytosis has preceded the evolution of apoptosis. Energy conservation could have offered the selective advantage for communal single-celled organisms to evolve mechanisms of programmed cell death. In turn, energy transfer morphed into 'information transfer' as the newly dead learned to instruct the cells that survive through the active production of heat-shock proteins and cytokines, and the delivery of antigenic peptides.

With specific consideration to cell death aiding in the maintenance of homeostasis, I contend that most have approached this matter from the standpoint of apoptotic cells needing to be 'non-inflammatory'. The difficulty with this argument is the identification of selective pressure for energy-dependent complex biological processes that involve many genes/gene families. It is interesting to consider how examples of these pathways, such as the binding of high mobility group box 1 (HMGB1) to hypo-acetylated DNA, might have evolved. If we consider that phagocytosis is the driving force in the evolution of apoptosis, we instead ask: what does hypo-acetylated DNA achieve that is of benefit to the phagocyte? One intriguing possibility is that such a modification would allow more efficient energy transfer in the form of nucleotides; HMGB1 might serve to facilitate this process further. Secondary to this event, an inflammatory role for this chromatin-binding protein might have evolved. As with all evolutionary arguments, this will be difficult to prove; nevertheless, a shift in our thinking with respect to the inflammatory nature of death is essential for the better understanding of life.

Conclusions

In considering death as an upstream point of immune regulation (BOX 2), I have attempted to approach the question of activation versus tolerance from the perspective of cells in resting peripheral tissue. Polly Matzinger has often remarked that we must add a new layer of cells to the question of immune regulation — normal cells in peripheral tissue, which signal to the immune system through the release of danger signals[65]. We now know that antigen derived from internalized apoptotic cells might be processed and presented by DCs for ligation by $CD4^+$ and $CD8^+$ T cells. As such, the dying cell is influencing immunity. With respect to the different types of cell death, I expect that as we uncover the intricacy of programmed cell death pathways, we will no longer think of necrosis versus apoptosis. Instead, it will be important to define the specific death effector pathways triggered, the class of proteins upregulated and the acetylation state of the DNA. Furthermore, I predict that we will identify molecules that are released by healthy cells and during homeostatic death that actively facilitate immunological tolerance. This added level of complexity will synergize with the growing insights from the field of clearance and the important role of receptor use in influencing immunity. Much work will be required to defend my contention that apoptosis versus necrosis is not the relevant immunological question. Instead, we should be concerned with the range of possible apoptotic deaths that a cell might undergo. In this way, we may decipher the complex and active signals that are transmitted between the newly dead cells and the phagocytes that are interested in gleaning information for protection of the living.

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The author declares that he has no competing financial interests.

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OPINION

NKT cells: what's in a name?

Dale I. Godfrey, H. Robson MacDonald, Mitchell Kronenberg, Mark J. Smyth and Luc Van Kaer

Recent years have seen so-called natural killer T (NKT) cells emerge as important regulators of the immune response. The existence of NKT-cell subsets, and other types of T cell that resemble NKT cells, is an ongoing source of confusion in the literature. This perspective article seeks to clarify which cells fall under the NKT-cell umbrella, and which might be best considered as separate.

The term 'NK T cells' was first published in 1995 (REF. 1) and was used broadly to define a subset of mouse T cells that share some characteristics with NK cells, particularly expression of the NK1.1 marker (Nkrp1c or CD161c). The term NKT cells is now well accepted and is applied to mice, humans and other species. However, the classification of NKT cells has always been complicated by the fact that most commonly used mouse strains (apart from C57BL/6) do not express the NK1.1 marker. Furthermore, as the field of NKT-cell research develops, it is becoming increasingly clear that the simplistic definition that NKT cells are NK1.1$^+$ T cells (even in C57BL/6 mice) is not only inaccurate, but also potentially misleading. This Opinion article seeks to clarify some of the problems associated with the definition of NKT cells, and to compare and contrast the different types of T cell that are often considered as being within the broader NKT-cell family. The article mainly focuses on NKT-cell subsets in mice, with occasional references to humans and other species where appropriate.

A brief history of NKT cells

Research carried out in many laboratories over the past two decades led to the discovery and definition of NKT cells (TIMELINE). In 1987, three separate groups published studies showing the existence of a distinct subset of αβ-T-cell receptor (αβ-TCR)$^+$ T cells in mice that expressed intermediate rather than high levels of TCR, with a two- to three-fold higher frequency of Vβ8 expression than conventional T cells, and that lacked expression of CD4 and CD8 accessory molecules[2–4]. General interest in these double-negative (DN) αβ-TCR$^+$ lymphocytes increased when it was discovered that they were a potent source of immunoregulatory cytokines, including interleukin-4 (IL-4), interferon-γ (IFN-γ) and tumour-necrosis factor (TNF)[5]. Around the same time, other groups reported on the existence of a subset of αβ-TCR$^+$ T cells that expressed NK1.1, which was previously considered to be only expressed by NK cells[6,7]. These NK1.1$^+$ T cells also expressed intermediate levels of TCR with a bias towards Vβ8.2 expression, and included two subsets — CD4$^+$ T cells and DN T cells. Similar to NK1.1$^+$αβ-TCR$^+$ DN T cells, NK1.1$^+$CD4$^+$ cells were also found to be a potent source of immunoregulatory cytokines[8,9]. Others had similarly found that subsets of CD4$^+$ T cells in the thymus produced large amounts of cytokines[10–12]. However, at the time that these studies with thymocytes and peripheral T cells were published, it was unclear whether they were all dealing with the same cell type. Considering that NK1.1$^-$CD4$^+$ thymocytes were not a potent source of cytokines[8], it was probable that most CD4$^+$, high cytokine-producing T cells in the thymus were included in the NK1.1$^+$ fraction. Collectively, these studies indicated the existence of a unique subpopulation of T cells, defined by NK1.1 expression, that were potent cytokine producers and therefore likely to be effective immunoregulatory cells.

Further evidence for the unique character of NK1.1$^+$αβ-TCR$^+$ cells came from observations that their development was independent of MHC class II expression, but required β_2-microglobulin (β_2m), although they did not express CD8 (REFS 13–15). This led to the realization that NKT cells are reactive to the MHC class-I-like molecule CD1d[16]. In addition to the marked bias in TCR Vβ-chain usage