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THE ROYAL SOCIETY

T-cell proliferation *in vivo* and the role of cytokines

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Unlike typical naive T cells, T cells with an activated ($CD44^{hi}$) memory phenotype show a rapid rate of proliferation *in vivo*. The turnover of memory-phenotype $CD8^+$ T cells can be considerably augmented by injecting mice with various compounds, including polyinosinic–polycytidylic acid, lipopolysaccharide and immunostimulatory DNA (CpG DNA). Certain cytokines, notably type I (α, β) interferons (IFN-I), have a similar effect. These agents appear to induce proliferation of $CD44^{hi}$ $CD8^+$ cells *in vivo* by an indirect process involving production of effector cytokines, possibly interleukin-15, by antigen-presenting cells. Although none of the agents tested induces proliferation of naive-phenotype T cells, IFN-I has the capacity to cause upregulation of surface markers on purified naive T cells. Depending upon the experimental conditions used, IFN-I can either inhibit or enhance primary responses of naive T cells to specific antigen.

Keywords: cytokines; proliferation; T cells; CpG DNA; interferons

1. INTRODUCTION

Relative expression of surface markers such as CD44 subdivides $CD4^+$ and $CD8^+$ T cells into distinct subsets of naive-phenotype ($CD44^{lo/int}$) and memory-phenotype ($CD44^{hi}$) cells (MacKay 1993; Sprent 1994, 1995; Zinkernagel *et al.* 1996; Dutton *et al.* 1998). In young animals, most T cells are naive, i.e. cells that have yet to make contact with specific antigen. These long-lived cells rarely divide and express a characteristic naive ($CD44^{lo/int}$) phenotype (Sprent 1993; Tough & Sprent 1994). When naive T cells respond to a foreign antigen, they proliferate and differentiate into a mixture of short-lived effector cells and long-lived memory cells; both cell types express a $CD44^{hi}$ phenotype, indicating a state of acute or chronic activation, and display a high rate of turnover (Tough & Sprent 1994). T cells with this phenotype form only a small proportion of the total in young animals, but become a dominant population with advancing age. Collectively, memory-phenotype T cells are thought to represent memory cells specific for the various environmental antigens encountered throughout life.

2. LONGEVITY OF MEMORY T CELLS

Immunological memory is usually long lived and, especially for viruses, generally lasts for the lifetime of the animal (MacKay 1993; Sprent 1994, 1995; Zinkernagel *et al.* 1996; Dutton *et al.* 1998). Why memory T cells have a prolonged life span is still unclear. Since memory T cells have a rapid turnover, the prevailing view is that the long-term survival of these cells requires some form of chronic stimulation. The nature of the stimuli involved, however, is a topic of considerable controversy. Some workers have argued that the protracted survival of memory T cells requires continuous contact with specific antigen, e.g. with small depots of the priming antigen bound to follicular dendritic cells in germinal centres (Gray 1993; Kundig *et al.* 1996). However, at least in the case of $CD8^+$ cells, memory T cells can survive for prolonged periods on adoptive transfer under conditions where contact with specific antigen is precluded (Hou *et al.* 1994; Lau *et al.* 1994; Mullbacher 1994). An alternative possibility is that memory cells survive via cross-reactive contact with various environmental antigens (Beverley 1990). If so, the survival of memory cells would be a stochastic process and would not apply to T cells that lacked specificity for environmental antigens. A third possibility is that memory cells are kept alive by recognition of self antigens, i.e. self peptides bound to major histocompatibility complex (MHC) molecules. This idea deserves consideration because memory-phenotype T cells are reported to disappear rapidly after transfer to MHC-deficient hosts (Tanchot *et al.* 1997).

3. STIMULI FOR PROLIFERATION

As for survival, the high turnover (rate of proliferation) of memory T cells is presumed to reflect some form of chronic stimulation. Here, the simplest idea is that memory cells are engaged in chronic responses to a spectrum of MHC-associated peptides. As mentioned above (§ 2), the peptides concerned could be derived from specific foreign antigens, cross-reactive environmental antigens or self antigens. Such stimulation via MHC–peptide

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complexes could be augmented by contact with various cytokines.

Since most memory T cells display a semi-activated phenotype and have higher levels of cytokine receptors than naive cells, it would seem quite likely that contact with stimulatory cytokines could contribute to the high turnover of memory T cells. To assess this idea, we have been investigating the capacity of various cytokines to amplify the turnover of the memory-phenotype T cells found in normal unimmunized mice.

4. T-CELL PROLIFERATION ELICITED BY TYPE I INTERFERONS

Our interest in the possible role of type I (α, β) interferons (IFN-I) in T-cell turnover stemmed from the finding that the T cells proliferating during infection with lymphocytic choriomeningitis virus and other viruses expressed high levels of Ly6C (Tough *et al.* 1996). Since Ly6C upregulation is reported to be under the sole control of IFN-I (Dumont & Coker 1986), it was conceivable that a proportion of the T cells proliferating during viral infection were stimulated non-specifically by the large amounts of IFN-I that are known to be released during viral infections (Buchmeier *et al.* 1980).

In favour of this possibility, injecting normal mice with a single dose of purified IFN-I (IFN-β) or polyinosinic–polycytidylic acid (Poly I:C), a strong inducer of IFN-I, caused a marked transient increase in the proliferation of memory-phenotype T cells (Tough *et al.* 1996) (figure 1). The proliferative response reached a peak at two to three days after injection and was largely restricted to memory-phenotype ($CD44^{hi}$) $CD8^+$ cells; proliferation of $CD44^{hi}$ $CD4^+$ cells and naive-phenotype ($CD44^{lo/int}$) T cells was minimal.

To assess whether T-cell proliferation following IFN-I injection required co-ligation of T-cell receptor (TCR) molecules, we tested whether $CD8^+$ cells could respond to IFN-I in β2-microglobulin (β2m)-deficient mice; in these mice, MHC class I expression is very low, thus minimizing contact of $CD8^+$ cells with MHC class I–peptide complexes. Using $\beta2m^{-/-}$ $CD8^+$ cells raised in bone marrow chimeras, it was found that $\beta2m^{-/-}$ $CD44^{hi}$ $CD8^+$ cells were strongly responsive to IFN-I (Poly I:C) after adoptive transfer to $\beta2m^{-/-}$ hosts (Tough *et al.* 1996). In light of this finding, responsiveness of $CD44^{hi}$ $CD8^+$ cells to IFN-I did not appear to depend upon co-ligation of the TCR.

The simplest explanation for the proliferation of $CD44^{hi}$ $CD8^+$ cells induced by IFN-I injection was that IFN-I stimulated T cells directly. However, this possibility was ruled out by the finding that IFN-I failed to cause proliferation of purified $CD44^{hi}$ $CD8^+$ cells *in vitro* (Zhang *et al.* 1998); in fact, under these conditions IFN-I had a strong anti-proliferative effect on T cells (see § 9). Based on these data, the *in vivo* effects of IFN-I were presumably indirect and reflected production of secondary cytokines, i.e. effector cytokines that acted directly on T cells. Currently, we have no direct evidence on the nature of the putative effector cytokines involved. As discussed below (§ 5), however, interleukin (IL)-15 has emerged as a likely candidate.

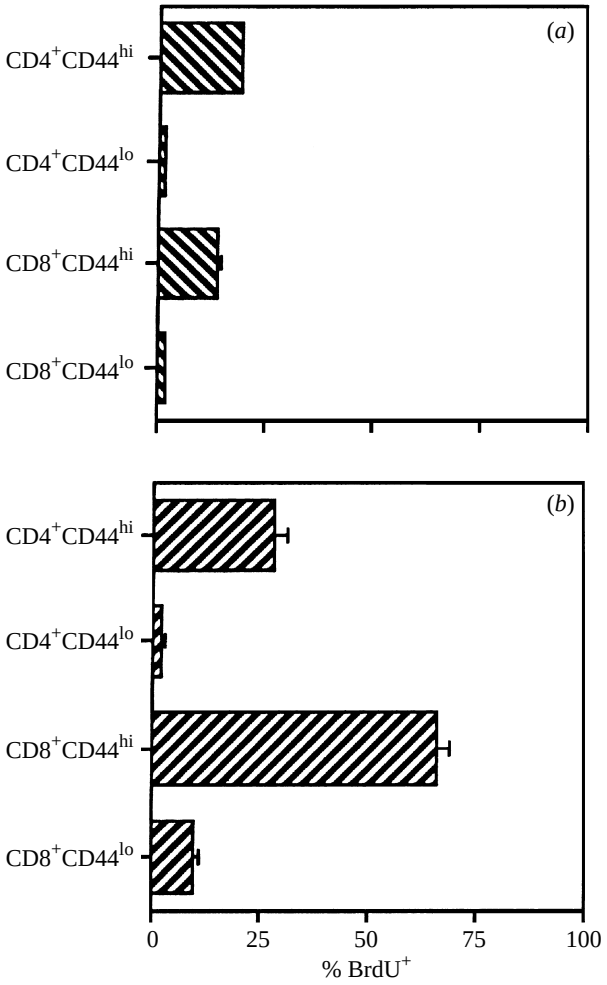

Figure 1. Poly I:C induction of T-cell proliferation *in vivo*. Thymectomized B6 mice were injected with 150 µl phosphate-buffered saline (*a*) or 150 µg Poly I:C (*b*) intraperitoneally, and given bromodeoxyuridine (BrdU) in their drinking water for three days. Pooled lymph node cells were stained for CD4 or CD8, CD44 and BrdU and analysed by flow cytometry. Data adapted from Tough & Sprent (1994).

5. IL-15 AS AN EFFECTOR CYTOKINE FOR IFN-I-INDUCED T-CELL PROLIFERATION

Since IFN-I-induced proliferation of $CD44^{hi}$ T cells *in vivo* is much more prominent for $CD8^+$ cells than $CD4^+$ cells, it follows that the receptors for the putative effector cytokine elicited by IFN-I would have to be expressed selectively on $CD44^{hi}$ $CD8^+$ cells. On this point, examining the expression of IL receptors IL-2Rα, β and γ on T-cell subsets revealed that one of these receptors, IL-2Rβ (CD122), was expressed at a much higher level on $CD44^{hi}$ $CD8^+$ cells than on $CD44^{hi}$ $CD4^+$ cells (Zhang *et al.* 1998) (figure 2). Since only two known cytokines, IL-2 and IL-15, bind to IL-2Rβ (Kennedy *et al.* 1998), these two cytokines were prime contenders for the effector cytokines driving IFN-I-induced T-cell proliferation *in vivo*.

Testing these two cytokines under *in vivo* and *in vitro* conditions appeared to exclude a decisive role for IL-2. Thus, although IL-2 did stimulate T-cell proliferation,

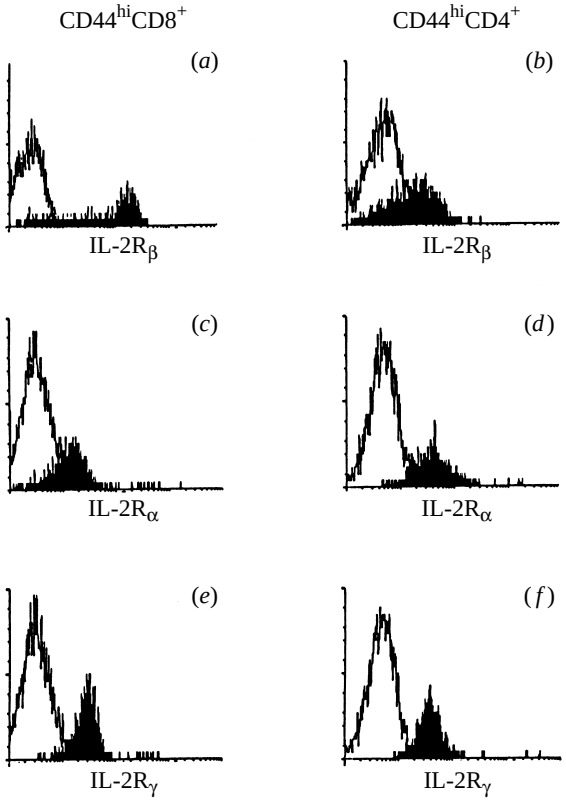

Figure 2. Expression of IL-2 receptor subunits on memory-phenotype T-cell subsets. Using three-colour staining, pooled lymph node cells from normal mice were stained with monoclonal antibodies (mAbs) specific for IL-2Rα, IL-2Rβ or IL-2R γ followed by mAbs specific for CD4, CD8 and CD44. Gated $CD4^+$ or $CD8^+$ cells were analysed for IL-2Rα, β or γ chain expression on $CD44^{hi}$ subpopulations. IL-2Rβ expression was much lower on $CD44^{lo/int}$ cells (not shown). Data adapted from Zhang *et al.* (1998).

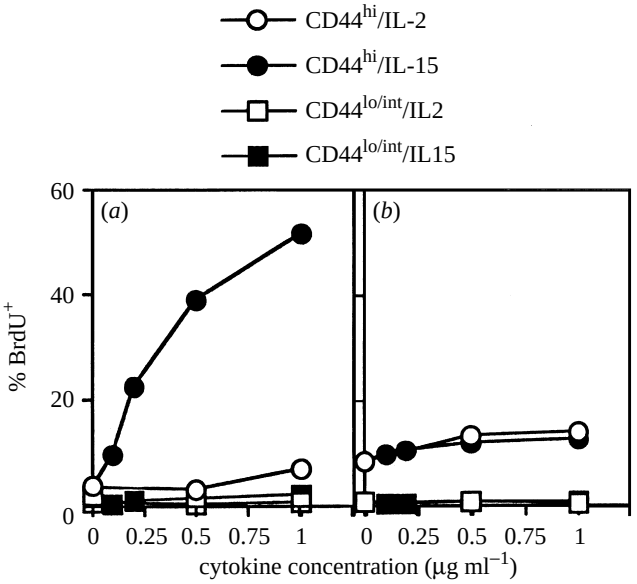

Figure 3. Effects of IL-2 and IL-15 on T-cell proliferation *in vitro*. Purified populations of $CD8^+$ (*a*) or $CD4^+$ (*b*) cells were cultured in the presence of various concentrations of cytokines for 24 h in the presence of BrdU (12.5 μg ml^{-1}). After removal of dead cells by Ficoll gradients, cells were stained for surface markers and BrdU incorporation and then analysed by fluorescence-activated cell sorting. The data show the percentage BrdU labelling of subsets of T cells expressing different levels of CD44. Data adapted from Zhang *et al.* (1998).

this effect of IL-2 was not selective for $CD8^+$ cells. By contrast, IL-15 was strongly stimulatory for $CD44^{hi}$ $CD8^+$ cells but had little activity on $CD44^{hi}$ $CD4^+$ cells (Zhang *et al.* 1998) (figure 3). Significantly, selective stimulation of $CD44^{hi}$ $CD8^+$ cells by IL-15 applied not only *in vivo* but also to purified T cells cultured *in vitro*. Hence, unlike IFN-I, IL-15 displayed direct effector function for $CD44^{hi}$ $CD8^+$ cells.

The notion that IFN-I induces T-cell proliferation *in vivo* via IL-15 production rests on the assumption that IFN-I is able to elicit IL-15 synthesis. Since many cell types, including macrophages (but not T cells), can synthesize IL-15 (Kennedy *et al.* 1998), we examined IL-15 messenger RNA (mRNA) expression by macrophages after short-term exposure to IFN-I *in vitro* (Zhang *et al.* 1998). In fact, IFN-I proved to be a strong inducer of IL-15 mRNA; several other stimuli, including IFN-γ, lipopolysaccharide (LPS) and immunostimulatory DNA (CpG DNA), shared this property.

Collectively, the above data make a strong case that IL-15 is an important effector cytokine for the proliferation of the $CD44^{hi}$ $CD8^+$ cells seen after IFN-I injection. However, the data are still largely indirect and definitive information on this issue will have to await studies with IL-15-deficient mice.

6. OTHER INDUCER CYTOKINES FOR PROLIFERATION OF $CD44^{HI}$ $CD8^+$ CELLS *IN VIVO*

Information on the capacity of other cytokines to induce T-cell proliferation *in vivo* is still sparse. However, preliminary work has shown that at least three other cytokines, namely IFN-γ, IL-12 and IL-18, share the capacity of IFN-I to induce strong proliferation of $CD44^{hi}$ $CD8^+$ cells under *in vivo* conditions (D. Tough, J. Sprent, X. Zhang and S. Sun, unpublished data). Interestingly, two of these cytokines, IL-12 and IL-18, also induce significant proliferation of $CD44^{hi}$ $CD4^+$ cells. The mechanisms involved here are still unknown.

7. T-CELL PROLIFERATION BY OTHER STIMULI

Like Poly I:C, a number of other compounds have the capacity to cause strong proliferation of $CD44^{hi}$ $CD8^+$ cells *in vivo*. These compounds include LPS (Tough *et al.* 1997) (figure 4), killed bacteria, CpG DNA (insect DNA) and synthetic oligodeoxynucleotides (ODNs) containing immunostimulatory CpG motifs (S. Sun, D. Tough, J. Sprent and X. Zhang, unpublished data). At least at the mRNA level, all of these agents are able to induce IL-15 production by macrophages, suggesting that, like IFN-I, these compounds induce T-cell proliferation via IL-15 production. Whether these compounds act as direct stimulators of IL-15, however, or function via prior production of IFN-I and/or other inducer cytokines is still unclear.

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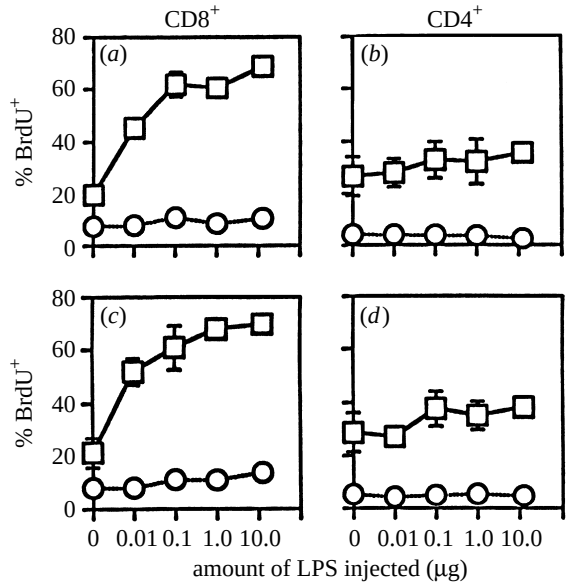

Figure 4. Effect of LPS injection on T- and B-cell proliferation *in vivo*. Graded doses of LPS were injected intravenously into B6 mice. Mice were immediately given BrdU in their drinking water, and lymph node (LN) (*a*, *b*) and spleen (*c*, *d*) cells were analysed three days later. The data show the percentage of BrdU labelling for $CD44^{hi}$ (squares) and $CD44^{lo}$ (circles) $CD8^+$ and $CD4^+$ cells. Data adapted from Tough *et al.* (1997).

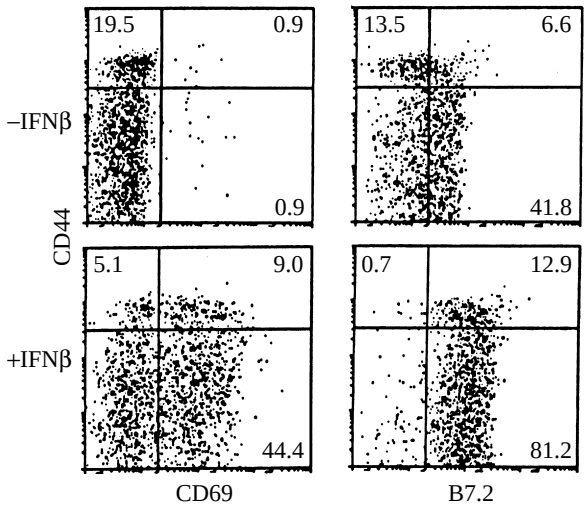

Figure 5. Upregulation of CD69 and B7–2 on purified $CD8^+$ cells in response to IFN-β *in vitro*. Purified $CD8^+$ cells (2×10^6 cells per well) were cultured with or without IFN-β (10 000 units ml^{-1}) overnight; cells were then triple-stained for CD8, CD44 and CD69 or B7–2. The data are shown gated for $CD8^+$ cells.

8. ACTIVITY OF IFN-I ON NAIVE T CELLS

Although *in vivo* proliferation of T cells induced by IFN-I and the above (see § 7) agents was largely restricted to $CD44^{hi}$ $CD8^+$ cells, we were surprised to find that these agents also caused partial activation (but not proliferation) of naive T cells. Thus, when mice were injected with LPS (Tough *et al.* 1997) or CpG DNA–ODNs (Sun *et al.* 1998*b*), the naive $CD4^+$ and $CD8^+$ T cells in these mice showed conspicuous upregulation of CD69, an activation marker and also several other cell-surface markers including B7–2. Similar findings applied to other cell types such as B cells and antigen-presenting cells (APC).

At least in the case of CpG DNA–ODNs, upregulation of surface markers on naive T cells was found to be APC dependent (Sun *et al.* 1998*b*), suggesting that T-cell stimulation was induced indirectly via production of cytokines by APC. Here, the notable finding was that upregulation of surface markers after injection of CpG DNA–ODNs was virtually undetectable in mice lacking receptors of IFN-I (Sun *et al.* 1998*b*). The implication, therefore, was that stimulation of naive T cells by CpG DNA–ODNs was mediated by IFN-I released by APC. If so, it followed that IFN-I would be able to act directly on naive T cells. In confirmation of this prediction, culturing purified APC-depleted naive T cells *in vitro* with IFN-I induced much the same pattern of surface marker upregulation as elicited by CpG DNA–ODNs supplemented with APC (Sun *et al.* 1998*b*) (figure 5); IFN-γ was ineffective.

9. OPPOSING EFFECTS OF IFN-I ON NAIVE T CELLS

As mentioned earlier (§ 8), upregulation of surface markers on naive T cells after culture with IFN-I was not associated with cell division. In fact, in line with the evidence that IFN-I has an anti-proliferative function (Grander *et al.* 1997), addition of IFN-I inhibited the capacity of T cells to mount *in vitro* proliferative responses following TCR ligation or exposure to stimulatory cytokines (S. Sun, J. Sprent, X. Zhang and D. Tough, unpublished data). IL-2 synthesis was not impaired, however, which suggests that IFN-I does not act by simply inhibiting intracellular signalling (Petricoin *et al.* 1997). Based on studies with cell lines, it would seem likely that the state of partial activation induced by IFN-I is associated with production of cell-cycle inhibitors (Grander *et al.* 1997). Whether this scenario applies to resting T cells has yet to be examined.

Interestingly, preliminary work showed that the anti-proliferative effect of IFN-I on naive T cells only occurred in the absence of viable APC, for example, when purified T cells were stimulated by cross-linked anti-TCR plus anti-CD28 mAbs (J. Sprent, X. Zhang, S. Sun and D. Tough, unpublished data). Thus, when T-cell responses were driven by peptides presented by viable APC, the effects of IFN-I were quite different. In fact, in this situation addition of IFN-I had the opposite effect and augmented the T-cell proliferative response; this property of IFN-I was not shared by IFN-γ. The implication therefore is that the presence of viable APC not only counters the anti-proliferative function of IFN-I but allows IFN-I to act as a powerful adjuvant. This latter function of IFN-I seems to predominate under normal physiological conditions *in vivo*. Thus, when naive TCR-transgenic T cells were exposed to specific peptide on adoptive transfer, T-cell proliferative responses to peptide were considerably enhanced by co-injection of IFN-I (S. Sun, J. Sprent, X. Zhang and D. Tough, unpublished data). CpG DNA–ODNs, i.e. compounds that lead to strong production of IFN-I, displayed a similar adjuvant function for naive T cells *in vivo* (Sun *et al.* 1998*a*).

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10. CONCLUDING COMMENTS

The data summarized above indicate that the effects of cytokines on T cells *in vivo* are complex and depend upon the activation status of T cells.

For memory-phenotype T cells, a wide variety of cytokines and cytokine-inducing agents induce selective stimulation of a subset of these cells, namely $CD44^{hi}$ $CD8^{+}$ cells. The majority of these cytokines do not act directly on T cells but appear to function by eliciting the production of effector cytokines, i.e. cytokines that are directly stimulatory for T cells. Currently, the identity of these effector cytokines has yet to be defined, but IL-15 has emerged as a likely possibility.

The biological significance of T-cell proliferation induced by IFN-I and other cytokines *in vivo* is still unclear. Since infectious micro-organisms often induce strong cytokine production, either by directly infecting host cells or via stimulatory products such as LPS and CpG DNA, many forms of infection may have the potential to cause non-antigen-specific proliferation of pre-existing memory cells specific for other antigens. Until recently, such 'bystander' stimulation of T cells was thought to be a prominent feature of typical immune responses, e.g. to viral infections (Tough *et al.* 1996). However, with the recent development of highly sensitive techniques for detecting antigen-specific T cells, it is now apparent that the majority of T cells proliferating in viral infections are specific for viral antigens (Butz & Bevan 1998; Gallimore *et al.* 1998; Murali-Krishna *et al.* 1998). Hence, the status of bystander T-cell proliferation during infection is now much less clear (Ehl *et al.* 1997; Zarozinski & Welsh 1997; Tough & Sprent 1998).

In view of the wide range of cytokines released during the immune response to viruses and other pathogens, it is difficult to envisage how pre-existing memory cells specific for other antigens would escape being subjected to bystander stimulation. Our suggestion of several years ago was that bystander stimulation might serve as a survival boost for memory cells (Tough *et al.* 1996). This idea has come under attack from the subsequent finding that bystander stimulation can actually shorten the survival of memory cells (Selin *et al.* 1996). Thus, exposing virus-A-primed mice to virus B was found to diminish memory for virus A. The physiological significance of this finding is unclear because it would predict that memory to a virus encountered early in life would rapidly wane through subsequent repeated contact with other viruses. Yet memory to viruses is usually lifelong, at least in mice.

One possible explanation for the finding that memory to virus A can be reduced by infection with virus B is that cytokine production elicited by some viruses is so intense that bystander stimulation is followed by T-cell death. By contrast, minor infection with viruses might lead to relatively low production of cytokines and thus cause only low-level bystander stimulation. Such 'gentle' stimulation of memory T cells could be beneficial and promote survival of the stimulated cells. We favour this idea because exposing murine $CD44^{hi}$ $CD8^{+}$ cells to a single dose of IFN-I caused the cells to proliferate briefly and then differentiate into long-lived resting memory cells (Tough *et al.* 1996). However, direct evidence on the relationship of the intensity of bystander T-cell proliferation and the subsequent fate of the stimulated cells is still lacking.

Whether bystander proliferation plays a role in the background proliferation of memory T cells in normal unimmunized animals is unclear. This is a difficult issue to address directly because it would entail depriving memory cells from contact with cytokines. Constructing such an experiment under *in vivo* conditions would be difficult. However, it is of interest that mice lacking an important receptor for IL-15, namely IL-15Rα, show a considerable reduction in memory-phenotype T cells (Lodolce *et al.* 1998). One might interpret this finding as indicating that depriving memory T cells from stimulation via IL-15 shortens the life span of the cells. However, the effects of deleting other cytokine receptors, notably IL-2Rα and IL-2Rβ, are quite different. Thus, in $IL\text{-}2R\alpha^{-/-}$ and $IL\text{-}2R\beta^{-/-}$ mice, memory-phenotype T cells are over-abundant and have an abnormally high turnover rate, thus leading to lymphadenopathy (Suzuki *et al.* 1995, 1997; Willerford *et al.* 1995; Ohteki *et al.* 1997; X. Zhang , J. Sprent, S. Sun and D. Tough, unpublished data). In light of these findings, the role of cytokines in controlling the normal 'background' turnover of memory-phenotype T cells is still largely obscure.

With regard to naive T cells, to date, we have seen no evidence that exposure to cytokines *in vivo* is able to induce proliferation of typical resting naive-phenotype T cells. However, it is notable that injecting mice with cytokine-inducing compounds such as LPS and CpG DNA–ODNs causes many cell types, including naive-phenotype T cells, to upregulate a variety of surface markers such as CD69 and B7–2 (Tough *et al.* 1997; Sun *et al.* 1998*b*). Based on studies with $IFN\text{-}IR^{-/-}$ mice, the upregulation of these markers does not reflect a direct action of LPS and CpG DNA–ODNs on T cells but stimulation of T cells by IFN-I released from APC. Thus, unlike LPS and CpG DNA–ODNs, IFN-I is able to induce upregulation of surface markers on APC-depleted purified T cells *in vitro* (Sun *et al.* 1998*b*). Whether other cytokines have this property has yet to be studied.

The physiological significance of the partial activation of naive T cells (CD69 and B7–2 upregulation) induced by IFN-I is still unclear. Under *in vitro* conditions, exposure to high concentrations of IFN-I in the absence of APC has a marked anti-proliferative effect on T cells; since IL-2 secretion is not impaired, the inhibition of proliferation may reflect induction of cell-cycle inhibitors. Whether IFN-I has a direct anti-proliferative action on T cells under *in vivo* conditions, however, is uncertain because, at least *in vitro*, the presence of viable APC counters the anti-proliferative effect. However, the protective effect of APC might not apply *in vivo* if T cells were subjected to prolonged exposure to high concentrations of IFN-I. On this point it is notable that some viral infections are associated with transient, generalized immuno-incompetence.

It is of interest that, in moderate doses, IFN-I has powerful adjuvant activity for T cells. Thus, as mentioned earlier (§ 9), exposure to IFN-I intensified the proliferative response of naive T cells to antigens presented by normal viable APC, both *in vitro* and *in vivo*. This property of IFN-I is shared by Poly I:C (Tough *et al.* 1996) and CpG DNA–ODNs (Sun *et al.* 1998*a*). How these agents augment primary responses of T cells is unknown,

although upregulation of co-stimulatory molecules on APC and/or release of stimulatory cytokines by APC are logical possibilities.

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