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IN VIVO ACTIVATION OF ANTIGEN-SPECIFIC CD4 T CELLS

Marc K. Jenkins[1], Alexander Khoruts[2], Elizabeth Ingulli[3], Daniel L. Mueller[2], Stephen J. McSorley[1], R. Lee Reinhardt[1], Andrea Itano[1], and Kathryn A. Pape[1]

[1]Department of Microbiology, Center for Immunology, University of Minnesota Medical School, Minneapolis, MN 55455; e-mail: marcj@mail.ahc.umn.edu, mcsor001@tc.umn.edu, reinhard@lenti.med.umn.edu, itano001@c.umn.edu, pape001@tc.umn.edu

[2]Department of Medicine, Center for Immunology, University of Minnesota Medical School, Minneapolis, MN 55455; e-mail: khoru001@tc.umn.edu, dmuell@lenti.med.edu

[3]Department of Pediatrics, Center for Immunology, University of Minnesota Medical School, Minneapolis, MN 55455; e-mail: ingul001@tc.umn.edu

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■ **Abstract** Physical detection of antigen-specific CD4 T cells has revealed features of the in vivo immune response that were not appreciated from in vitro studies. In vivo, antigen is initially presented to naïve CD4 T cells exclusively by dendritic cells within the T cell areas of secondary lymphoid tissues. Anatomic constraints make it likely that these dendritic cells acquire the antigen at the site where it enters the body. Inflammation enhances in vivo T cell activation by stimulating dendritic cells to migrate to the T cell areas and display stable peptide-MHC complexes and costimulatory ligands. Once stimulated by a dendritic cell, antigen-specific CD4 T cells produce IL-2 but proliferate in an IL-2–independent fashion. Inflammatory signals induce chemokine receptors on activated T cells that direct their migration into the B cell areas to interact with antigen-specific B cells. Most of the activated T cells then die within the lymphoid tissues. However, in the presence of inflammation, a population of memory T cells survives. This population is composed of two functional classes. One recirculates through nonlymphoid tissues and is capable of immediate effector lymphokine production. The other recirculates through lymph nodes and quickly acquires the capacity to produce effector lymphokines if stimulated. Therefore, antigenic stimulation in the presence of inflammation produces an increased number of specific T cells capable of producing effector lymphokines throughout the body.

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INTRODUCTION

The adaptive immune response is the result of interactions between foreign substances and the host's antigen receptor–bearing lymphocytes and antigen-presenting cells (APC). These interactions take place in several complex microenvironments within the body and play out over a period of several weeks, with the end result being elimination of the foreign substance. The complexity of the immune response is so daunting that reductionist approaches have been necessary to understand the interacting parts. In vitro culture systems, cloned cell lines, purified lymphokines, and synthetic peptide antigens have contributed to our current understanding that CD4 T cells produce lymphokines and proliferate when their T cell antigen receptors bind to an APC displaying the appropriate peptide–class II MHC molecule and costimulatory ligands (1, 2).

However, reductionist approaches may now be limiting our ability to understand and manipulate the in vivo immune response. The long-term T cell clones and transformed cells lines often used in vitro may not behave like naïve or memory T cells that participate in the in vivo immune response. Disruption of lymphoid tissues to produce the single cell suspensions required for in vitro cultures destroys the spatial relationships between T cells and APC that exist in vivo and separates the cells from factors produced by the lymphoid stroma. In vitro cultures cannot be used to study the critically important process whereby T cells migrate from lymphoid sites of initial activation to nonlymphoid sites of antigen deposition. Perhaps the greatest limitation of in vitro culture systems, however, is that they do not replicate the effect that inflammation has on the in vivo T cell response. It has been known for many years that adjuvants, and the inflammatory mediators that they induce, influence the quality of the T cell response to foreign antigen. Injection of foreign antigen with an adjuvant induces robust humoral and cell-mediated immune responses, whereas injection of foreign antigen alone does not (3). Furthermore, injection of purified foreign antigen in the absence of inflammation induces a state of unresponsiveness to subsequent immunization with antigen plus adjuvant (4, 5). To date, the fundamental capacity of inflammation to dictate T cell immunity or tolerance cannot be replicated in vitro.

Recently, several approaches have been developed that allow the physical tracking of T cells of known peptide-MHC specificity within the body during in vivo immune responses. These approaches have shed light on in vivo antigen presentation, T cell proliferation, death, migration, tolerance induction, and memory cell generation. This review focuses on these new findings with an emphasis on unique features of the in vivo response that were not appreciated in cell culture studies. In addition, the steps in the in vivo CD4 T cell activation process that are regulated by inflammation are discussed.

METHODS FOR TRACKING ANTIGEN-SPECIFIC CD4 T CELLS IN VIVO

Past attempts to study the T cell response in vivo relied on functional tests to enumerate the number of cells present. Limiting dilution analyses of antigen-driven proliferation or cell-mediated cytotoxicity produced estimates of the frequency of antigen-specific T cells present at various times after introduction of antigen into the host (6). The limitation of this approach is that T cells must undergo many cell divisions and survive for several weeks in culture to be scored in the assay. Furthermore, the cells must perform the function being measured. Antigen-specific T cells that do not survive well in vitro, or are not, for example, cytotoxic, are missed with this type of assay. The frequency with which antigen-specific T cells produce a given lymphokine in response to in vitro stimulation with antigen has been determined by the enzyme-linked immunospot assay (7). The sensitivity of this assay is comparable to flow cytometric detection of antigen-specific T cells using fluorochrome-labeled peptide-MHC multimers when the T cells under study have been primed in a such a way that all antigen-specific T cells produce the lymphokine being measured (8). However, this is not always the case, and in these situations, enzyme-linked immunospot assay underestimates the frequency of antigen-specific T cells (9).

The solution to this problem has come from methods that allow physical detection of T cells based solely on TCR specificity. The most direct method relies on fluorochrome-labeled, multimeric peptide-MHC complexes. One version of this approach involves refolding soluble, empty, class I MHC molecules with a single antigenic peptide. The peptide-MHC complex is then biotinylated and mixed with a streptavidin-labeled fluorochrome to produce a tetramer (10). This tetramer binds to CD8 T cells that express an appropriate TCR. The strength of this approach is that it can theoretically measure all potentially responsive T cells in the normal repertoire. A weakness is that the frequency of T cells specific for most peptide-MHC complexes in naïve individuals is below the limit of detection of flow cytometry (8, 11, 12). Thus, peptide-MHC multimers cannot currently be used to study the immune response before clonal expansion occurs. Another weakness is that peptide–class II MHC tetramers are more difficult to prepare because the peptide must be covalently attached to the class II MHC molecule (13).

A second method is based on the knowledge that ~70% of the CD4 T cells that respond to the pigeon cytochrome c peptide 81-104 in mice that express H-2 I-E^k possess a TCR containing a characteristic TCR-Vα chain and CDR3 region on the TCR-Vβ chain (14). Because the system is used to track the normal T cell repertoire, it has the same strengths and weaknesses as peptide-MHC multimer-based detection: The entire repertoire of pigeon cytochrome c peptide-I-E^k-specific CD4 T cells can theoretically be tracked but, because of the infrequency of naïve precursors, only after clonal expansion.

One way to solve the technical problem of the infrequency of naïve T cells with a single specificity is adoptive transfer of naïve TCR transgenic T cells into syngeneic normal recipients (15). This maneuver produces a traceable naïve T cell population of known peptide-MHC specificity within the recipient, comprising 0.5–1% of cells in the secondary lymphoid organs. The transferred cells can be distinguished from those of the recipient with antibodies specific for the TCR clonotype or an allelic marker such as Thy 1 or CD45. One advantage of this method is that the earliest events in T cell activation in vivo can be studied because the antigen-specific T cells are abundant enough to be detected by flow cytometry or immunohistology before clonal expansion. A potential disadvantage is that even though only a small number of T cells is transferred, the resulting frequency of antigen-specific T cells is still higher than normal. Although all of the effects of this elevated frequency are unknown, the kinetics and relative magnitude of clonal expansion and loss reported for transferred T cells after in vivo exposure to antigen are identical to those described for endogenous T cells tracked by the two methods described above (15).

LOCATION OF NAÏVE T CELLS

Most studies of the earliest events in the T cell activation process in vivo have focused on the secondary lymphoid organs (lymph nodes, spleen, and Peyer's patches) because a variety of methods have shown that naïve CD4 T cells are found primarily, if not exclusively, in these tissues (16–18). Naïve CD4 T cells are further restricted within secondary lymphoid organs to the T cell–rich areas known as the paracortex in the lymph nodes and Peyer's patches, and the periarteriolar lymphoid sheath (PALS) in the spleen (19) (Figure 1). The restriction to lymph nodes is explained by the fact that naïve T cells express a unique set of receptors, which bind ligands that are only expressed on the specialized blood vessels of the lymph nodes known as high endothelial venules (HEV). For example, naive T cells use CD62L for rolling on vessel walls, and CC chemokine receptor (CCR) 7 for integrin activation and extravasation (20). HEV are the only blood vessels in the body that display the ligands for these receptors (Glycam-1, CD34, SLC, and ELC) (20). Naïve T cells move from the blood into the spleen because all blood contents are emptied directly from terminal branches of the central arteriole into marginal sinuses and then the red pulp (19). The T cells then move from the red pulp into the PALS by a poorly understood CD62L-independent, G protein–dependent mechanism (21). Once in the PALS or paracortex, naïve T cells remain there in part because they express CCR7 and sense the SLC and ELC chemokines that are produced in the T cell areas (22). After spending about one day in the T cell areas, naïve T cells leave the lymphoid tissue in which they reside and return to blood to enter a different lymphoid tissue.

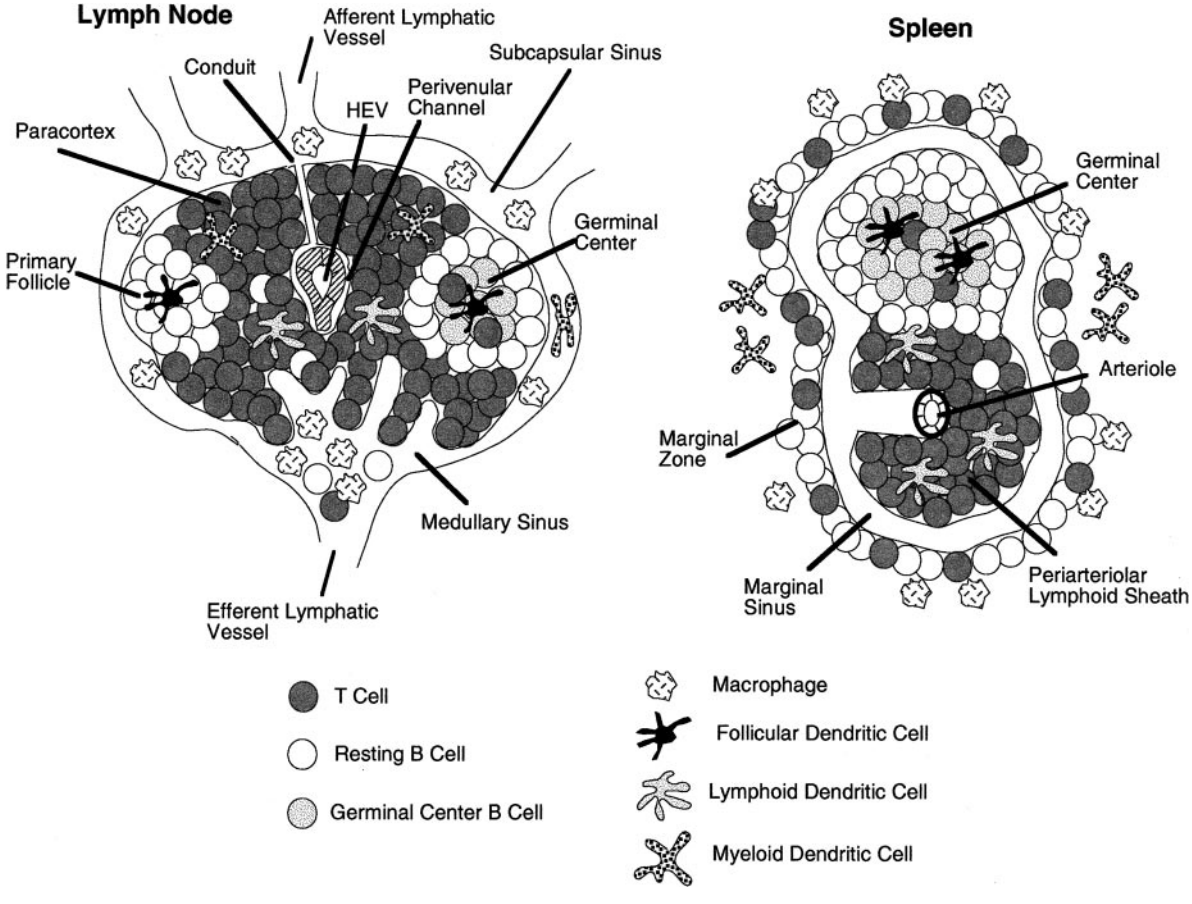

Figure 1 Schematic views of cross-sections through a lymph node and one splenic white pulp cord.

IN VIVO ANTIGEN PRESENTATION TO NAÏVE T CELLS

The restriction of naïve T cells to the T cell areas of secondary lymphoid organs implies that the APC that initiate the primary immune response must also be located there. This contention is supported by the finding that the earliest signs of activation in naïve T cells in vivo can be detected within an hour of antigen injection (23), a time when the cells are still in the T cell area (24). By far the most abundant class II MHC-expressing cell type in the T cell area is the dendritic cell (25). The other potential class II MHC-expressing APC for CD4 T cells, B cells, and macrophages are located outside of the T cell areas; B cells reside in follicles and macrophages in the subcapsular and medullary sinuses of lymph nodes and the red pulp and marginal zone of the spleen (19). Thus, the anatomy, and a wealth of in vitro experiments demonstrating their potency as APC for naïve T cells (25), suggests that dendritic cells are the APC that initiate in vivo T cell responses.

We obtained physical evidence supporting this hypothesis by staining lymph node sections from recipients of ovalbumin peptide-I-A^d-specific TCR transgenic T cells with a pan-dendritic cell-specific anti-CD11c antibody and an anti-clonotypic antibody specific for the transferred T cells, each labeled with a different fluorochrome (26). In the absence of ovalbumin, the transferred T cells were intermingled with dendritic cells in the paracortex of the lymph node, and many of the T cells were in physical contact with a dendritic cell. Within 16 h of subcutaneous injection of ovalbumin, the number of contacts and the average size of the contact area between the transferred T cells and dendritic cells increased three- to fourfold over the basal level. Antigen-dependent increases in interaction between the antigen-specific T cells and macrophages or B cells were not observed at these early times because these cells were not present in the T cell areas. Similarly, MacLennan and colleagues found that antigen-stimulated CD4 T cells first entered the cell cycle when associated with dendritic cells in the T cell areas (27). These results are consistent with the hypothesis that the initial antigen presentation to naïve CD4 T cells in vivo is carried out exclusively by dendritic cells. In vitro studies where cells from dissociated splenic tissue are used as APC do not replicate this phenomenon because B cells are by far the most abundant APC under these conditions.

Dendritic Cell Migration

The dendritic cells that were found interacting with antigen-specific T cells in the paracortex could have acquired soluble antigen from incoming lymph. Anatomic evidence suggests that this is probably not the case, however. Lymph is carried from tissues via afferent lymphatic vessels into the subcapsular sinus of a connected lymph node (19) (Figure 1). Cells appear to be capable of squeezing between the cells that make up the floor of this sinus and entering the regions occupied by T and B cells (28). Surprisingly, however, soluble molecules do not easily flow from

the subcapsular sinus into the lymphocyte-rich areas of the lymph node. Tracers injected into afferent lymphatic vessels appear in the subcapsular sinus and in thin conduits that run through the lymphocyte-rich areas but not in the lymphocyte-rich areas themselves (28).

The inaccessibility of the T cell area to lymph-borne antigen raises the possibility that antigen is carried from the site of antigen deposition into the T cell area by a migrating dendritic cell. The T cell areas of the lymph nodes are occupied by two types of dendritic cells, one that expresses CD11b and is derived from the myeloid lineage and another that expresses DEC-205 and CD8α and is derived from the lymphoid lineage (29). Both types of dendritic cells probably originate in nonlymphoid tissues as one of several immature precursors. For example, the skin contains epidermal Langerhans cells and monocytes, both of which have been shown to migrate from nonlymphoid tissues into the T cell areas of the draining lymph node (30, 31). Langerhans cells express DEC-205 (32), whereas monocytes that migrate to the T cell areas acquire some of the phenotypic characteristics of dendritic cells, including high-level expression of MHC and B7, but lack DEC-205 and CD8α (31). These markers and results from several gene-targeting experiments (33, 34) indicate that lymphoid dendritic cells are derived from migrating Langerhans cells, whereas myeloid dendritic cells are derived from migrating monocytes. The finding that cells with the phenotype of myeloid dendritic cells can be grown in vitro from monocyte precursors in the presence of GM-CSF and IL-4 further supports the latter possibility (35). Preliminary results from our studies suggest that the dendritic cells that are interacting with antigen-specific CD4 T cells early after subcutaneous injection of intact ovalbumin are myeloid dendritic cells. This could be related to the fact that monocytes have better access to antigen in the subcutaneous tissue than do Langerhans cells or dermal dendritic cells. In contrast, antigens that enter the epidermis are probably carried to the T cell area and presented by Langerhans cells.

In contrast to the lymph nodes, tracer studies have shown that the T and B cell areas of the spleen are freely accessible to soluble antigen shortly after intravenous injection. Staining with a monoclonal antibody specific for a peptide-class II MHC complex derived from hen egg lysozyme (HEL) showed that most B cells in the follicles produced peptide-MHC complexes from HEL within several hours (36). However, peptide-MHC complexes were not detected on most of the dendritic cells in the T cell areas at this early time. Such complexes appeared eventually, first in the outer PALS and then throughout the PALS by 24 hr after HEL injection. This process is greatly enhanced in the presence of LPS-induced inflammation (37), as is the migration of myeloid dendritic cells from the marginal zone into the PALS (38). Therefore even in the absence of a physical barrier to free antigen, antigen presentation in the splenic T cell areas may be carried out by dendritic cells that acquire antigen outside of the T cell area. This implies that the resident lymphoid dendritic cells of the spleen are inefficient at antigen uptake or processing, perhaps as a result of prior maturation (39). However, antigen presentation by resident lymphoid dendritic cells cannot be ruled by this approach because it

is unlikely that peptide-MHC-specific antibodies can detect the low number of peptide-MHC complexes that can trigger a T cell.

Dendritic Cell Maturation

The movement of dendritic cell precursors from nonlymphoid tissues or the marginal zone into the T cell areas is greatly enhanced by inflammation (38). Dendritic cell migration in the context of inflammation is associated with a functional maturation process involving changes in antigen processing and T cell stimulation potential (40). Freshly isolated Langerhans cells, or monocyte-derived dendritic cells grown in vitro with GM-CSF and IL-4, efficiently engulf particles including dying cells and large volumes of extracellular fluid and store the ingested material in unprocessed form in MIIC vesicles. Exposure to inflammatory mediators, such as LPS, causes these immature dendritic cells to produce peptide–class II MHC complexes and shuttle these complexes into CIIV vesicles that eventually fuse with the plasma membrane (41). Inflammation also reduces the turnover of peptide–class II MHC complexes on the surface of dendritic cells (42). This maturation process is likely a mechanism of adjuvant action. Inflammation caused by adjuvants would lead to much greater antigen presentation because many immature dendritic cells would migrate from the tissue of antigen deposition into the T cell area, and in the process mature to display more stable and abundant peptide-class II MHC complexes.

CLONAL EXPANSION

Naïve CD4 T cells proliferate in the T cell areas shortly after recognition of peptide-MHC complexes on dendritic cells (24, 27). This proliferation is evidenced by an increase in the number of antigen-specific CD4 T cells within the relevant secondary lymphoid organs, 3–7 days after antigen injection (14, 27, 43, 44). The magnitude of the clonal expansion is much greater if antigen is administered with an adjuvant (43). For example, naïve antigen-specific T cells increase 10–20-fold in number in the lymphoid tissue 3 days after subcutaneous injection of soluble ovalbumin, and 20–100-fold after injection of ovalbumin plus LPS (45) or unmethylated CpG-containing DNA (46).

Role of CD28

The effect of adjuvant-induced inflammation on T cell proliferation correlates with a preceding effect on lymphokine production. Approximately three times as many antigen-specific naïve CD4 T cells produce IL-2 in vivo 10 hr after subcutaneous injection of antigen plus LPS as do so after injection of antigen alone (47). The antigen-stimulated T cells must express CD28 to experience the enhancing effect of LPS on early IL-2 production and later clonal expansion (47). The requirement for CD28 is probably related in part to the capacity of LPS to stimulate B7 expression in dendritic cells (38) and to direct B7 molecules into the

CIIV vesicles containing peptide-MHC complexes (41), ensuring that the TCR and CD28 ligands are localized on the surface.

Role of IL-2

The correlation between clonal expansion and IL-2 production made it reasonable to suspect that the in vivo proliferation of antigen-stimulated T cells would be critically dependent on IL-2. This hypothesis was also supported by the finding that T cells from IL-2-deficient mice proliferate much less well in vitro than do T cells from normal mice in response to optimal TCR stimulation (48). Surprisingly, however, the TCR-driven clonal expansion of CD4 T cells lacking IL-2 (49) or components of the IL-2 receptor (50, 51) is minimally or not at all impaired in vivo. Moreover, the ability of LPS to enhance clonal expansion of naïve antigen-specific CD4 T cells is preserved in the absence of IL-2 (47). Therefore, other signals or growth factors must be capable of driving CD4 T cell proliferation in vivo, although IL-2 may contribute. As noted below, IL-2 plays an important role in the elimination of activated T cells. The dual function of IL-2, as both a T cell growth factor early in the response and a death factor later, may make it difficult to reveal the growth factor activity of IL-2 in IL-2-deficient animals.

Role of Inflammatory Cytokines

IL-1 or TNF-α mimic the enhancing effect of LPS on the in vivo expansion of antigen-stimulated T cells (45). These inflammatory cytokines may mediate this effect indirectly through CD28. Adjuvant molecules are recognized by pattern recognition receptors (52) on cells of the innate immune system, for example macrophages, at the antigen injection site, causing the release of TNF-α and IL-1. These cytokines may stimulate B7 expression on antigen-presenting dendritic cells, resulting in a greater level of CD28 signaling in interacting T cells. Anti-CD40 antibodies may mediate adjuvant effects by a similar B7-dependent mechanism (53). However, it is also likely that CD28 costimulation is involved in a direct effect of IL-1 or TNF-α on CD4 T cells. Support for this possibility comes from in vitro experiments that show that the proliferation of highly purified CD4 T cells in response to plastic surfaces coated with TCR and CD28 ligands is augmented by IL-1 (54, 55). Since IL-1 could not act on an APC in this experiment, it must have acted directly on the T cells.

FOLLICULAR MIGRATION

One of the most important functions of CD4 T cells is to recognize peptide-MHC complexes on antigen-specific B cells and provide help for antibody production. Because naïve T and B cells are anatomically separated from each other in the secondary lymphoid organs, one or both cell types must move if interaction is to take place. Recent work suggests that T and B cells specific for epitopes from

the same antigen both move from their starting locations to meet at the border between the T and B cell areas (24, 56, 57). Nahm and coworkers provided the first evidence for the T cell movement by showing that CD4 T cells expressing MBP-specific TCR V segments appeared in the follicles of mice immunized with MBP (58). The basis for this phenomenon was identified in several tracking studies that showed that antigen-specific CD4 T cells migrate from the T cell area into the B cell–rich follicles, after first proliferating in the T cell area (43, 44, 59). The migration of activated CD4 T cells into follicles only occurred in mice that were injected with antigen and adjuvant (43, 45). This phenomenon is explained by regulated expression of chemokine receptors. Naïve T cells express CCR7, which is specific for chemokines produced by stromal cells in the T cell areas (SLC and ELC), but not CXCR5, which is specific for BLC produced by follicular stromal cells (22). Cyster and coworkers showed that CXCR5 expression is induced on antigen-specific T cells several days after in vivo exposure to antigen and adjuvant, but not antigen alone (60). The requirement for adjuvant may be explained by the findings of Lane and colleagues that CXCR5 induction and follicular migration are dependent on signals through CD28 and OX40, the ligands for which (B7 and OX40 ligand) are induced on dendritic cells by inflammation (61). Induction of CXCR5 on antigen-specific CD4 T cells correlates with a gain in the ability to migrate in response to BLC and loss of responsiveness to SLC and ELC (60). The predicted effect of these changes in chemokine receptor expression would be loss of retention in the T cell areas and directed movement toward the follicles.

ELIMINATION OF ACTIVATED T CELLS

The number of antigen-specific T cells in the lymphoid tissues falls dramatically after the peak of clonal expansion. Much of the loss must be due to cell death because the body would soon fill up with lymphocytes if this were not the case. However, there is confusion about the molecular basis for death because of conflicting results on the effects of death receptors and survival proteins. For example, the loss of antigen-stimulated T cells from the lymphoid tissue after the peak of clonal expansion has been shown to be Fas-dependent, bcl-2-insensitive in some studies (62) and Fas-independent, bcl-2-sensitive in others (63). Abbas and colleagues have proposed that these discrepancies are explained by the duration of antigen presentation (64). If antigen is presented transiently, perhaps because only a single antigen injection was given or the antigen under study has a short in vivo half-life, then the loss is caused by growth factor withdrawal. This type of apoptosis occurs via a Fas-independent, caspase-9-dependent pathway, which is antagonized by bcl-2 (65). This is a reasonable scenario because in vivo lymphokine production ceases at least one day before the beginning of the loss phase (47). If antigen is presented chronically, because of repeated injection or expression by the host, then Abbas and colleagues propose that activation-induced cell death occurs (64). This type of apoptosis is dependent on Fas and is poorly

inhibited by bcl-2 (65). This scenario is plausible because chronic activation causes expression of Fas on T cells (66). In addition, a death pathway involving Fas could explain the paradoxical death-promoting effects of IL-2 because IL-2 prevents the activation of FLICE inhibitor protein, which normally inhibits Fas signaling (67).

It should be noted that agreement on these scenarios has not been reached. Using an ex vivo assay, Marrack and coworkers showed that superantigen-stimulated T cells die by a mechanism that involves internal production of reactive oxygen species, but not Fas, TNF receptors, or caspases (68). These investigators argue that reactive oxygen species damage mitochondrial membranes, leading to metabolic dysfunction and death.

The loss phase of the response is another site of adjuvant action. In the absence of adjuvant-induced inflammation, the loss of antigen-specific T cells from the lymphoid tissues after the peak of clonal expansion is nearly complete. In contrast, many more cells survive the loss phase after injection of antigen or superantigen plus adjuvants such as LPS or IL-1 (43, 45, 69). This sparing effect can be induced by injection of LPS 24 hr after superantigen injection (69) and induced equally well in normal and CD28-deficient mice (70). Because lymphokine production by antigen-stimulated T cells is CD28-dependent (47), it is unlikely that this is the target of this late adjuvant effect. It is possible that LPS promotes survival by protecting T cells from the toxic effects of reactive oxygen species by some unknown mechanism.

GENERATION OF MEMORY CELLS

Affinity Maturation

The antigen-specific T cells that survive the loss phase after exposure of the host to antigen and adjuvant are responsible for immunological memory (71). In several systems where endogenous CD4 T cells were tracked, the antigen-specific cells from the secondary response expressed a restricted set of TCRs compared with those that participated in the primary response (14, 72). In this case, T cells from the secondary response possessed higher TCR affinities for peptide-MHC complexes derived from the immunogen than cells from the primary response (73). These results have led to the conclusion that the T cell response undergoes affinity maturation as T cells with the highest-affinity TCRs survive preferentially over T cells with lower affinities.

Acquisition of Effector Lymphokine Production Potential

The antigen-specific CD4 T cells that remain in the lymphoid tissues several weeks after exposure to antigen and adjuvant or microbes have phenotypic characteristics of memory T cells (74). They have divided many times and express low levels of CD45RB and high levels of CD44 and LFA-1. In addition, the antigen-experienced

CD4 T cells produce lymphokines more rapidly than naïve T cells and at lower doses of antigen. Antigen-experienced CD4 T cells also produce a broader set of lymphokines than do naïve T cells. Naïve CD4 T cells produce large amounts of IL-2 and TNF-α when stimulated by antigen in vivo but little or no effector lymphokines such as IFN-γ, IL-4, or IL-5. In contrast, antigen-experienced T cells from immunized hosts acquire the capacity to produce effector lymphokines depending on the cytokines present during the period of initial stimulation. In vitro studies have shown that naïve T cells that are stimulated with antigen in the presence of IL-12 or IFN-α differentiate into cells capable of producing the Th1 lymphokines IFN-γ and lymphotoxin upon recall, whereas cells that are stimulated in the presence of IL-4 become cells that produce the Th2 cytokines IL-4, IL-5, IL-6, and IL-10 (75). In vitro, this differentiation process can be driven to an extreme in which the T cells produce only the Th1 set or the Th2 set (76). Memory T cells resembling polarized Th1 or Th2 cells are induced in vivo in some immune responses usually involving chronic infections. For example, antigen-specific CD4 T cells from Leishmania-infected C57BL/6 mice produce primarily Th1 lymphokines, whereas cells from infected BALB/c mice produce Th2 lymphokines (77). However, in most immune responses including those in humans, memory T cells display complex patterns of lymphokine production that are not easily categorized as Th1- or Th2-type (78). The degree of heterogeneity in effector lymphokine production exhibited by memory T cells probably reflects the mixture of inflammatory cytokines produced by the innate immune system in response to the adjuvant present during the primary response. For example, naïve CD4 T cells are likely to acquire the capacity to produce IFN-γ in response to priming with antigen plus LPS or during Gram-negative bacterial infections because LPS is a strong inducer of IL-12 production in vivo (79, 80). Other microbial adjuvants may stimulate cells of the innate immune system to produce IL-12 and IL-4, causing the population of memory T cells to acquire Th1 and Th2 lymphokine production potential.

The important role that inflammatory cytokines play in T cell proliferation and differentiation may help explain why administration of antigen in the absence of inflammation induces immune tolerance. The few antigen-specific CD4 T cells that remain in the lymphoid tissues several weeks after initial exposure to antigen without inflammation also have phenotypic characteristics of memory T cells (23), and have divided in the past (23, 81). However, unlike memory cells produced by priming with antigen plus adjuvant, these antigen-experienced T cells do not produce IL-2 or effector lymphokines (23, 81, 82), with the possible exception of IL-10 (83), when challenged with antigen. The IL-2 production defect may be related to antigen presentation by the $B7^{low}$ dendritic cells that are present in uninflamed lymphoid tissues. The T cells might receive signals through CTLA-4 under these conditions because CTLA-4 has a higher affinity for B7 than CD28. CTLA-4 signaling has been shown to be critical for the induction of T cell anergy in vivo (82). This may come about because CTLA-4 inhibits T cell proliferation. In vitro experiments have shown that T cells are unable to produce IL-2 shortly

after stimulation through the TCR, and that proliferation is required for recovery from this defect (84–86). CTLA-4-mediated inhibition of proliferation would be expected to prevent this recovery, leaving the T cells in a hyporesponsive state. The effector lymphokine production defect observed in CD4 T cells after exposure to antigen in the absence of adjuvant is almost certainly related to the fact that the T cells recognized antigen in an environment devoid of IL-12 or other differentiating cytokines.

Migration

Memory T cells also differ from naïve T cells in their tissue distribution and circulation pattern (74). Mackay and colleagues found that most of the T cells that enter afferent lymphatic vessels express surface molecules typical of memory cells (87). Because lymphatic vessels receive cells from nonlymphoid tissues, this result suggests that some memory T cells reside in these sites. In addition, Lefrancois and coworkers showed that many antigen-stimulated CD8 T cells enter the lamina propria of the gut during the primary response and remain there in an activated state for many days (88). Recent work from our group, in which antigen-specific CD4 T cells were tracked by whole-body immunohistology, showed that nonlymphoid tissues (especially liver, lungs, and gut) are the major reservoir of antigen-experienced T cells after the loss phase of the primary response induced by intravenous injection of antigen plus adjuvant (18). The movement of antigen-experienced T cells into nonlymphoid tissues is another site of adjuvant action because very few antigen-stimulated T cells moved into nonlymphoid tissues in response to antigen alone. The antigen-experienced CD4 T cells that were present in nonlymphoid tissues after immunization with antigen plus adjuvant produced more IFN-γ after in vivo rechallenge with antigen than did the cells present in the lymph nodes of the same animals. Together these results are consistent with a model in which the antigen-specific CD4 T cells that achieve the highest level of differentiation (defined by IFN-γ production potential) leave the secondary lymphoid organs and enter the nonlymphoid tissues. This movement is probably facilitated by the induction of adhesive ligands on vascular endothelial cells by inflammatory cytokines produced in response to the adjuvant (89, 90). Because acquisition of IFN-γ production potential is related to prior exposure to IL-12 (75) and cell division history (91), it is possible that the T cells that enter the nonlymphoid tissues are those that experienced the highest IL-12 concentration and/or divided the most in the lymphoid tissues.

Evidence for the existence of a nonlymphoid tissue–seeking memory T cell population in humans has also been reported. Lanzavecchia and coworkers showed that CD45RA-negative, and thus antigen-experienced, T cells in the blood are comprised of two populations, one expressing CCR7 and the other lacking CCR7 (92). The CD45RA−, CCR7+ cells express high levels of CD62L and low levels of cutaneous lymphocyte antigen, LFA-1, and $\alpha 4\beta 1$ integrin. In contrast the CCR7− cells express low or variable levels of CD62L and high levels of cutaneous

lymphocyte antigen, LFA-1, and $\alpha 4\beta 1$ integrin. These expression patterns suggest that CD45RA−, CCR7+ cells circulate through lymph nodes, whereas CCR7− cells are excluded from lymph nodes and circulate through nonlymphoid tissues. The CCR7− cells produced IFN-γ, IL-4, and IL-5 rapidly when stimulated with anti-CD3 in vitro, whereas the CD45RA−, CCR7+ cells did not. However, CD45RA−, CCR7+ cells lost CCR7 after 10 days of in vitro stimulation with anti-CD3 and acquired the capacity to produce effector lymphokines. This result, and the finding that CCR7− cells had shorter telomeres (and thus had divided more often), suggest that CD45RA−, CCR7+ cells give rise to CCR7− cells. Together, these results suggest a scenario in which naïve T cells give rise to CD45RA−, CCR7+ cells that retain lymph node homing, which upon further stimulation give rise to CCR7− cells that circulate between blood and nonlymphoid tissues. Support for this idea comes from Bell and colleagues who identified two functionally distinct memory cell subsets in the rat (93).

CCR7 may also play a role in T cell migration within the lymphoid tissues in the mouse. Chaplin and colleagues reported that in vitro–differentiated Th2 cells migrated to the border between the T and B cell areas, whereas Th1 cells localized to the T cell areas of the spleen after adoptive transfer and immunization with antigen (94). Th1 cells expressed CCR7 and Th2 cells did not, and this difference played a role in migration behavior because transduction of the Th2 cells with CCR7 caused them to localize to the T cell areas. These results suggest that Th2 cells are good B cell helpers in part because they are not tethered by CCR7 in the T cell area and can migrate to the B cell areas. However, this model does not account for the capacity of Th1 cells to help B cells switch to IgG2a production (95) nor explain the fact that human IFN-γ-producing memory cells do not express CCR7 (92).

Persistence of the Memory Phenotype

The temporal stability of the phenotypic changes that occur in antigen-experienced CD4 T cells is controversial. On the one hand, in vitro–differentiated Th1 and Th2 cells retain their original lymphokine production patterns for months after transfer into naïve recipients (96). Similarly, Stockinger and coworkers found that antigen-specific CD4 T cells retained the $CD44^{high}$ phenotype and the capacity to produce IFN-γ for months under conditions where antigen was only presented for a brief period (97). On the other hand, work by other investigators has shown that $CD45RB^{low}$ cells revert to the naïve $CD45RB^{high}$ phenotype (98, 99) and that the enhanced helper function of CD4 memory T cells is lost over time (100). We found that naïve antigen-specific CD4 T cells became LFA-1^{high} within several days of immunization with antigen plus adjuvant but reverted to an LFA-1^{low} phenotype 10 weeks later, or within one week after transfer into naïve recipients, and reverted to some of the functional behaviors of naïve T cells (101). These results are consistent with the possibility that the expression of memory-phenotype homing receptors, and the nonlymphoid tissue-homing pattern determined by these receptors, are retained in CD4 T cells only as long as antigen is present. This may

be a major difference between T cell subsets because the memory phenotype and function of CD8 T cells are maintained in the absence of antigen (102, 103).

MODEL OF THE PRIMARY CD4 T CELL RESPONSE

Figure 2 shows a speculative model for the series of events that occur in naïve T cells during a primary immune response in the lymph nodes after subcutaneous injection of antigen plus adjuvant. This is the type of response that generates effector lymphokine-producing memory cells and is induced by microbes because they contain foreign proteins and molecules with adjuvant properties.

Adjuvant molecules are recognized by pattern recognition receptors on cells of the innate immune system at the antigen injection site, causing the release of TNF-α and IL-1. These cytokines signal the local tissue dendritic cells or monocytes to leave the tissue and migrate via an afferent lymphatic vessel to the draining lymph node after first ingesting antigen. During the migration process, these cells mature to produce peptide-MHC complexes from the ingested antigen and deliver these to the cell surface along with newly synthesized B7 molecules. After arriving at the lymph node, the dendritic cells crawl through the floor of the subcapsular sinus and present peptide-MHC complexes to naïve antigen-specific CD4 T cells in the T cell area. The T cells produce high levels of IL-2 and an unknown T cell growth factor, and they proliferate. This proliferation occurs in an IL-12-rich environment due to IL-12 produced by dendritic cells in response to the adjuvant. Many of the proliferating T cells die in the T cell area, but the anti-apoptotic effect of the adjuvant ensures that some survive. Of the survivors, those that divided the most and experienced the highest concentration of IL-12 lose CCR7, gain P-selectin ligand, and acquire the capacity for rapid IFN-γ production. Antigen-activated T cells that do not achieve this threshold number of cell divisions, or IL-12 concentration, remain CCR7+ and acquire rapid IL-2 production potential, but not the capacity for IFN-γ production. After leaving the lymphoid tissues during the primary response, the CCR7+ cells recirculate through lymphoid tissues like naïve T cells. In contrast, the CCR7− cells are excluded from lymph nodes and remain in the blood or enter nonlymphoid tissues that express P-selectin. As long as residual antigen is present to drive the survival of CCR7− cells, then a second exposure to antigen will result in rapid production of effector lymphokines at the site of antigen entry either by CCR7− cells that happen to reside in that tissue or by CCR7− cells that are rapidly recruited from blood. As antigen is cleared from the body, CCR7− cells die or revert to the CCR7+ phenotype; in either case only CCR7+ memory cells remain. If antigen enters the body during this phase, then CCR7+ cells will be activated in the lymphoid organs and rapidly differentiate into CCR7−, effector lymphokine-producing cells capable of migrating to the site of antigen deposition. This process would be more efficient than the primary response because CCR7+ memory cells could achieve effector lymphokine production faster than naïve T cells, and extrinsic factors such as antibodies from the primary response would facilitate antigen presentation (101, 104).

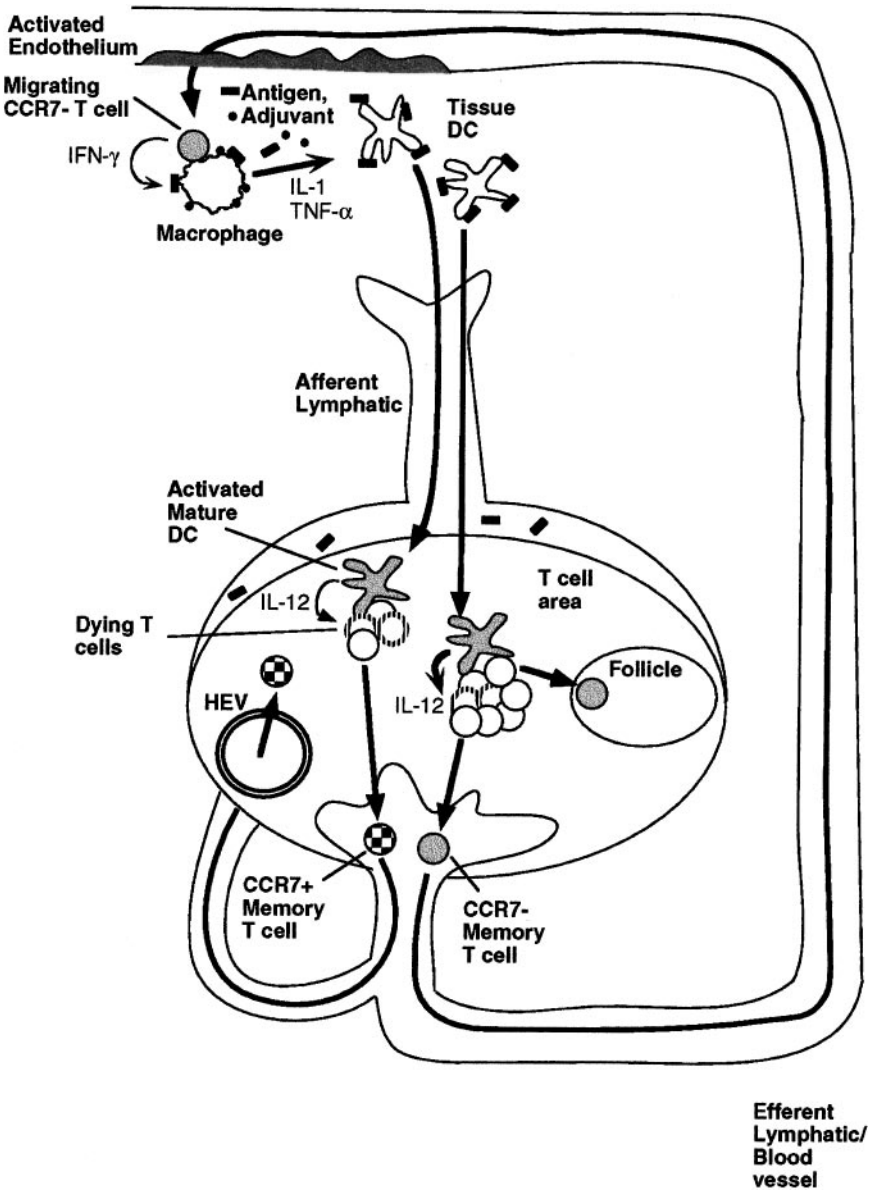

Figure 2 Diagrammatic representation of a productive primary CD4 T cell response to antigen in the presence of adjuvant-induced inflammation.

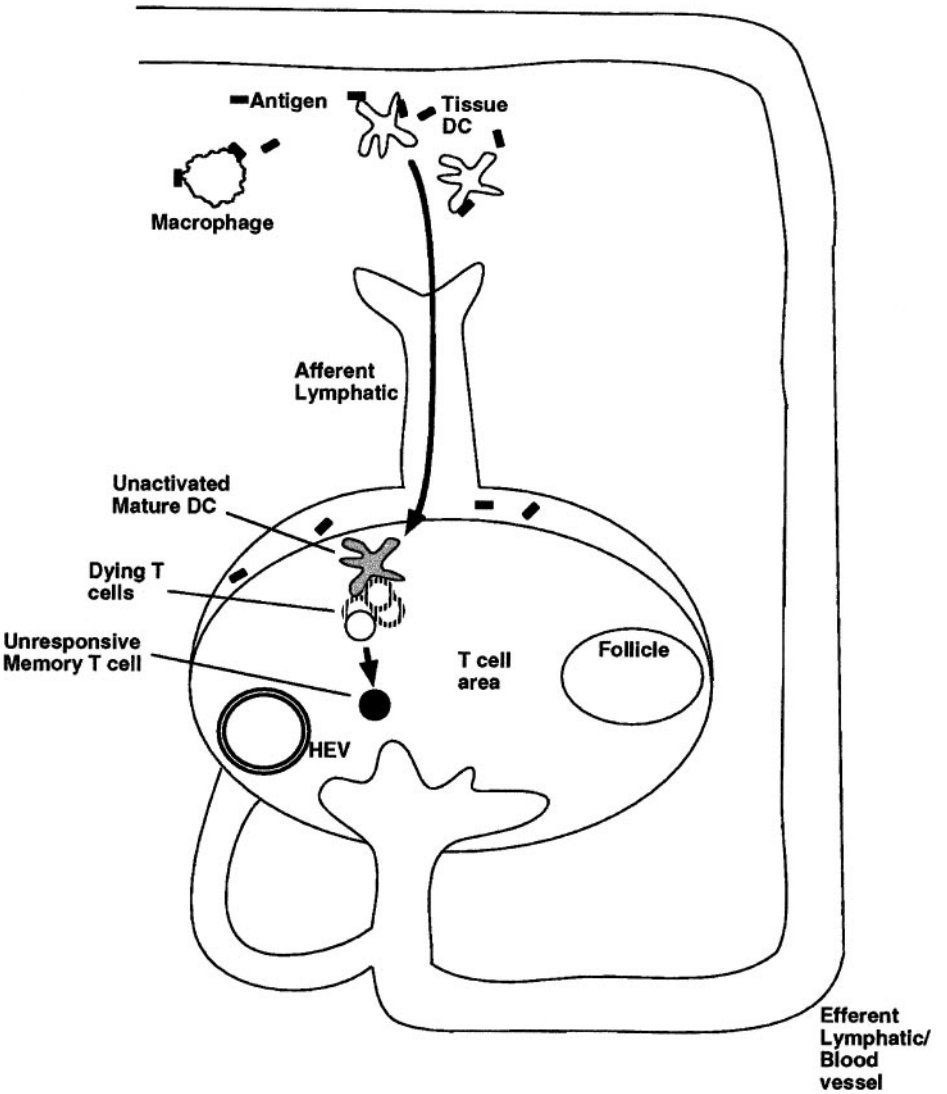

Figure 3 Diagrammatic representation of an unproductive CD4 T cell response to antigen in the absence of inflammation.

A very different outcome would result from antigen presentation in the absence of inflammation (Figure 3). Based on the aforementioned model, presentation of an injected antigen would be relatively inefficient because it would depend on the low level of dendritic cell migration that occurs under noninflammatory conditions, or the small amount of antigen that leaks across the subcapsular barrier

to be taken up by lymphoid–tissue resident dendritic cells. In either case, antigen presentation would be carried out by dendritic cells that do not express high levels of costimulatory ligands. Lack of the anti-apoptotic effects of inflammatory cytokines would cause most of the T cells to die. The minimal signaling through CD28, IL-1 receptor, and receptors for differentiating cytokines such as IL-12 and IL-4, experienced in the primary response would limit IL-2 and effector lymphokine production potential in any memory cells that survived. The combination of death of most of the expanded antigen-specific T cell population and the functional defects in the survivors could explain the induction of peripheral tolerance by antigen administration in the absence of inflammation.

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