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CD4+ T cell memory and antigen-specific cell frequency.

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CD4+ T CELL MEMORY AND ANTIGEN-SPECIFIC CELL FREQUENCY

by

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Title CD4+ T Cell Memory and Antigen-Specific Cell Frequency

Although immunological memory is characterized by both an increase in the frequency of antigen-specific T cells and a qualitative change in the pattern of their subsequent response, it is not clear which of these components is more significant in the overall enhanced response to secondary stimulation. To address this question for the CD4+ T cell response, DO11.10 TCR Tg T cells were adoptively transferred to normal syngeneic mice that were immunized with the relevant peptide. After the initial expansion of TCR Tg T cells, the size of the subsequent memory population of T cells was approximately the same as the size of the starting population, independent of the number of TCR Tg cells initially transferred. This result was not due to redistribution of memory cells into non-lymphoid tissues, although the relative frequency of antigen-specific T cells in these sites was increased after immunization. The fraction of the antigen-specific TCR Tg cells that responded by production of either IL-2 or IFN-γ *in vitro* was substantially higher after immunization. Thus, the increased frequency of functionally responsive T cells was primarily due to a higher fraction of responding T cells, rather than a substantial increase in the absolute number of antigen-specific CD4+ TCR Tg T cells.

Adoptive transfer experiments were also carried out using the OT-II and OT-I model systems. The results of the OT-II experiment confirmed that expanded popula-

tions of CD4+ memory cells do not persist *in vivo*. Unexpectedly, CD8+ OT-I adoptive transfer cells failed to persist as an expanded memory population.

DEDICATION

I dedicate this dissertation to my parents, who were and are my first teachers.

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DISCUSSION

LIST OF ABBREVIATIONS

AT	Adoptive transfer
ATR	Adoptive transfer recipient
CD	Cluster of differentiation
CFSE	5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester
FITC	Fluorescein-isothiocyanate
IFN	Interferon
IL	Interleukin
LN	Lymph node
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
OVAp	Chicken ovalbumin peptide (residues 323-339)
pMHC	Peptide-MHC
SA	Streptavidin
T	Thymocyte-derived
TCR	T cell receptor
Tg	Transgenic

INTRODUCTION

The Peripheral Thymocyte-derived Cell From 1961 to the Present

The history of peripheral T-cell biology can arguably be divided into three eras—functional, cellular, and molecular. The functional era began in 1961 when J.F.A.P. Miller published his article "Immunological Function of the Thymus" in *The Lancet* (1). Over the next 10 to 15 years the role of the thymus and thymus-derived lymphocytes in the immune response began to emerge.

However, the regulation behind this function remained unclear. In 1973 Rosenthal and Shevach published their finding that T lymphocytes were capable of mounting a response against a soluble antigen only after presentation of that antigen by histocompatible macrophages (2). Zinkernagel and Doherty's papers appeared in 1974 (3,4) and put forth the idea that T cells respond against target cells which bear a self H-2 gene product that has been altered in some way by the antigen. These findings provided a correct, precise theory of the target of the T cell response rather than simply observing alloreactivity among cells from different strains of mice. This new understanding opened the door to experiments aimed at understanding cellular events in T cell activation rather than simply observing the functions which result from that activation.

Over approximately the next 20 years, the period that can be regarded as the cellular era, a great deal was learned about the effector immune response and the pathways through which it acts. Advances in molecular biology facilitated the identification of

numerous effector and regulatory cytokines, and the availability of monoclonal antibodies allowed firm identification of the cell types involved. During this period, much of the immunology community spent their efforts on defining and characterizing different populations of cells. The view of the role of T cell was refined from a broad functional level to the level of knowing many of its effector response capabilities.

However, the molecular basis for the specificity behind these responses did not fully emerge until 1996, when the exact structure of the T cell antigen receptor and its precise relation to the major histocompatibility complex (MHC) was revealed through crystallographic studies (5,6). Also in 1996, soluble, peptide-loaded MHC reagents were developed that enabled the identification of peptide-specific populations of T cells (7). This advance began the molecular era of peripheral T-cell biology, and the ability to study T cells progressed from the population level to the level of the single, antigen-specific cell.

Advances in science are sometimes rapidly incorporated into mainstream thinking. Other times, incorporation is slower and possibly incomplete. The accomplishments listed above mark changes in how the immunology community thought about T cells. With each of these accomplishments, dominant ideas from the previous era were modified to account for new information. However, for cluster of differentiation 4 expressing (CD4+) T cells, the incorporation of new information into the collective schema has, on at least one point, been incomplete.

Prior to the ability to physically identify antigen-specific cells, assays for T-cell function were the best surrogate for T-cell frequency. Eventually this surrogate came to be mistaken for the actual frequency of antigen-specific T cells. This error occurred de-

spite numerous papers published in the mid-1970s that focused on the functional heterogeneity of T cells (8-15), and it resulted in the widespread perception that an increase in the number of antigen-specific cells is a large component of T cell memory. The data are now clear that this perception is correct as it pertains to CD8+ T cell memory. The data do not support the same conclusion in regards to CD4+ T cells, nor do they expressly disprove it. This underappreciated uncertainty surrounding the contribution of increased antigen-specific cell frequency to CD4+ T cell memory is the focus of this dissertation.

Cellular Immunological Memory

The Early Days

An awareness of immunological memory existed as early as the Plague of Athens in 430 B.C., at which time Thucydides reported that survivors of the plague were "never attacked twice--never at least fatally" (16). In the early 1700s Lady Mary Wortley Montagu brought the practice of variolation, which consisted of scratching particles from a small pox sore into the skin of an uninfected person, from present day Turkey to England. The result of variolation was generally a much attenuated infection that conferred lifelong immunity against the virus. In 1796 Edward Jenner variolated a young boy with material from a cow pox lesion and several weeks later variolated him again with material from a small pox lesion. No clinical symptoms resulted from the small pox variolation, and the practice was soon rechristened "vaccination" (17).

By the 1950s the scientific community had learned a great deal more about immunology. The perception of immunity was that it "rises during recovery, reaches a peak in late convalescence, and falls gradually in succeeding months"(18). The protective ef-

fects which result from a resolved infection were considered to be mediated mainly by circulating antibodies. A vague idea of "cellular antibodies" was associated with immunity to malaria and syphilis, though it was thought to be short-lived (18).

The Cellular Basis of Immunity

In 1962, James Gowan reported that small lymphocytes initiate the immune response (19). Earlier reports by another group had shown that the transfer of popliteal lymph node cells from rabbits that received injections of bacilli in the foot pad to uninjected rabbits resulted in detectable levels of antibody several days following cell transfer (20), but the occurrence of antibody in the cell transfer recipient rabbit was not fully accredited to the lymph node cells. Between 1962 and 1966, the observation was made that cell proliferation was linked to antibody formation following a secondary antigenic challenge (21,22). The origin of these proliferating cells remained uncertain until Gowans and Uhr immunized rats with ΦX174, and then waited up to 15 months before transferring thoracic duct lymphocytes into irradiated recipient rats (23). Cell transfer recipient rats which remained unimmunized after transfer had negligible levels of anti-ΦX174 antibodies. Immunization of the experimental group resulted in high titers of virus-specific antibody compared to the antibody levels in rats that received control cells from unimmunized mice. At that time, this experiment was the most definitive demonstration of the lymphocyte as the instrument of immunological memory.

The lymphocyte was still poorly characterized, but the mystery surrounding it was rapidly receding. In 1966 Claman *et al.* discovered that transfer of thymus-derived and marrow-derived cells into an irradiated host synergistically improved the antibody re-

sponse (24). That same year, Cooper *et al.* were carrying out experiments on the effect of irradiation on bursectomized and thymectomized chickens (25,26). By the end of the decade the distinction was drawn between T cells and B cells (27,28).

Cellular Immunology

Having attained a basic awareness of the two arms of the adaptive immune systems, the immunology community was prepared to pursue the characterization of those two arms. In 1969, the discovery of the theta isoantigen, later to be named CD90, as a marker of thymus-derived lymphocytes was the first step in being able to physically identify lymphocytes independently of their function (29). It also made possible the depletion of T cells from the lymphocyte population while leaving the B-cell population intact.

In 1970, immunological memory was recognized to involve "heightened response, earlier appearance of a detectable response, [and] more rapid development of peak values" (30). All of these characteristics remain correct by today's standard, but the understanding of how the cells of the immune system interact to achieve immunological memory was almost completely unknown. One central question was, for example, if B cells or T cells carried immunological memory.

In 1971, Miller and Sprent answered this question by transferring lymphocytes from fowl immunoglobulin G immunized mice into irradiated, non-immunized mice (31). The experimental design also allowed them to measure the response generated by primed and unprimed T cells in combination with primed and unprimed B cells. Sprent and Miller reported that removal of the primed T-cell population greatly reduced but did not abolish the antibody response that followed immunization of adoptive transfer recipient

mice. This effect could be overcome by transferring ten times as many unprimed T cells. The effect of the removal of primed B cells, however, could not be corrected by the transfer of more cells. The result led Sprent and Miller to conclude "that both T and B cells carried memory and that cell collaboration occurred in the secondary response"(31). It also left them with two possible explanations of the general mechanism underlying T cell memory:

> Since normal T cells can substitute for T cells from primed mice, albeit only when larger numbers are used, it might be concluded that memory in T cells involves a quantitative change in that population, i.e., an increase in the number of T cells reactive to the antigenic determinants concerned. The possibility of a qualitative change in T cells is not, however, excluded by the present results (31).

It also became clear in the early 1970s that T cells had more than one function. In 1970 Raff published a paper on the "role of thymus-derived lymphocytes in the secondary humoral immune response in mice" (32). Later that year, thymus-derived lymphocytes were also discovered to have cytotoxic capabilities after being "sensitized to alloantigens" (33,34). Over the next several years numerous papers were published detailing the functional heterogeneity of T cells (8-15). Included among these functional heterogeneities were the effector responses.

Major advances in understanding the activities of T cells were not accompanied by similar advances in the understanding of their specificity. As mentioned earlier, Zinkernagel and Doherty's work showed that T cells respond to antigen plus self MHC, but nothing was known of the molecular specifics involved in this recognition. As a result, the identification of antigen-specific T cells came to rely completely on their function. The qualitative differences between memory and naïve cells revealed a clear increase in the number of antigen-*reactive* cells present in the memory response. However,

the increase in numbers of antigen-reactive cells does not equate with an increase in the number of antigen-specific cells. In 1971 Sprent and Miller simply did not have access to an experimental system that would allow for making a distinction between antigen-reactive and antigen-specific cells. The methods developed in the 1970s and 1980s to measure the frequency of antigen-reactive cells would lead to a firm entrenchment of the idea that increases in absolute numbers of antigen-specific CD4+ T cells are a major component of CD4+ T cell memory despite a lack of data that explicitly supports that conclusion.

CD4+ T Cell Frequency Determination

All methods used to measure the frequency of antigen-specific T cells fall into either of two categories, functional or physical. Functional methods include limiting dilution analysis of various elements of the T-cell response and also direct measurement of the frequency of cytokine-producing cells. Physical methods require identification of the antigen-specific T cells independent of their function. This method relies on the ability to identify antigen-specific T cells either by their T cell receptor (TCR) or in some model systems by other genetic markers.

Functional Measures of T-Cell Frequency

The bulk of the early published experiments investigating antigen-reactive T-cell frequency obtained their results through limiting dilution analysis (35-39). These studies measured T-cell frequency on the basis of several functions, including proliferation in response to antigen, the ability to effect B cell help, and later Interleukin-2 and Inter-

feron-γ production. The Poisson distribution is the undergirding statistic for this measure of frequency. When a cell type is present at limiting dilution, the response being measured fails to occur in 37% of the replicate conditions. The derived frequency value reflects the frequency of "precursor" cells present in the culture, which give rise to the response measured following stimulation.

Direct measurements of cytokine production, principally ELISPOT and flow cytometry, are now more commonly used to estimate antigen-reactive CD4+ T cell frequencies than are limiting dilution analyses. The ELISPOT assay was originally developed to detect enterotoxin producing *E. coli* (40). The method was soon adapted to measure the frequency of antibody-producing cells in a population (41) and later, after the necessary antibodies became available, to measure the frequency of cytokine-producing cells (42). In this method, cells are plated on top of a nitrocellulose membrane that has been coated with an anti-cytokine antibody. Cells are activated to produce cytokine, and the nitrocellulose-bound antibodies capture the cytokine upon secretion from the cell. Enzyme-labeled antibodies specific for another epitope of the cytokine molecule are later applied to the membrane and developed with the appropriate substrate. The number of spots present on the membrane after development indicates the number of cytokine-producing cells present in the culture.

Detection of intracellular cytokines by flow cytometry offers several advantages over the ELISPOT method. Foremost among these is the ability to observe the response of defined populations of lymphocytes rather than the mass response of the bulk culture. In this method, cells that have been labeled with fluorochrome-conjugated antibodies spe-

cific for cell-surface markers are permeabilized and then incubated with fluorochrome-conjugated, cytokine-specific antibodies before analysis by flow cytometry.

While these methods are useful for measuring the frequency of cells that will respond in a given assay, they neglect the heterogeneity of T-cell populations. For example, all CD4+ T cells specific for a given antigen will not make IL-2 in response to stimulation with that antigen (43). Additionally, using these techniques to measure the frequencies of antigen-*specific* CD4+ T cells presupposes that the level of response within the antigen-specific population is the same for memory and naïve cells. True measurement of antigen-specific CD4+ T cell frequencies requires certain physical identification of the antigen-specific cells independent of their function.

Physical Measures of T-Cell Frequency

Antigen-specific T cells can be directly identified independently of their function by two different methods. In adoptive transfer, TCR transgenic (Tg) model systems, cells may be identified by expression of some genetic marker that distinguishes them from congenic host lymphocytes. In infectious systems, the means of identification is often dependent on recognition of specific TCR using fluorescently labeled peptide-MHC multimers.

Numerous TCR transgenic model systems have been developed. These systems have relied largely on known V beta usage by the antigen-specific TCR for identification following cell transfer into non-transgenic hosts. The presence of host cells that are not specific for the antigen under study but share V beta usage with the antigen-specific cells somewhat diminish the utility of this system. Recently some TCR transgenic mice have

been bred onto SCID-/- and RAG-/- backgrounds to express a different CD90 or CD45 allele from their parent strain. In these systems, all T cells harvested from the TCR transgenic donor animal are specific for a single peptide and can be identified on the basis of CD90 or CD45 alloexpression from endogenous T cells in the recipient animal. The DO11.10 TCR transgenic system is unique in that it relies on a monoclonal antibody KJ1-26.1 (44) that recognizes the chicken ovalbumin peptide 323-339 (OVAp)+I-Ad-specific TCR to identify antigen specific T-cells (45,46).

Altman and colleagues were the first to employ MHC tetramer complexes to characterize polyclonal populations of antigen-specific T cells at normal biological frequencies (7) when they compared HIV- and influenza-specific CD8+ T cells from HIV infected individuals. Tetramers have subsequently been used most notably in the mouse lymphocytic choriomeningitis virus infection model (47,48). MHCII tetramers proved more difficult to develop (49) but have been used to study the CD4+ T cell response to influenza hemagglutintin (50) and hepatitis C virus (51). The ability to study the T cell response starting from normal numbers of antigen-specific cells is a major advantage over the TCR transgenic adoptive transfer model systems; however, the inability to detect antigen-specific cells prior to challenge limits the usefulness of tetramers in directly measuring the expansion of cell populations. Additionally, tetramers identify a polyclonal population of antigen-specific T cells rather than a single clone of cells (52). T cell recognition of antigen occurs across a range of avidities, thus tetramers may fail to detect low-avidity, antigen-reactive cells that could potentially play an important role in the response.

Both approaches of physically measuring antigen-specific cells have provided insights that were unattainable using functional assays alone. In the case of CD8+ T cell biology, the results from tetramer experiments have confirmed older results, which showed an increase in the number of antigen-specific cells present following resolution of an antigenic challenge. Similar results for CD4+ T cells have not been published.

Qualitative Differences in Memory and Naïve Cells

Despite uncertainty about the contribution of increased numbers of antigen-specific cells to CD4+ T cell memory, some of the phenotypic and qualitative changes that occur in antigen-specific CD4+ T cells when they transition into memory cells have been documented.

The memory status of CD4+ T cells is usually correlated with increased expression of CD44 (53,54) and decreased expression of CD45RB (55,56) and CD62L (57). While this phenotype has been useful in identifying populations of memory CD4+ T cells, the stability of expression of these markers is not absolute, and populations of memory cells may revert to a naïve phenotype (58).

The functional differences discovered between memory and naïve CD4+ T cells confirm the "possibility of a qualitative change" that Sprent and Miller considered in 1971 (31). Specifically, these qualitative changes include faster entry into the cell cycle and faster expression of IFN-γ than naïve cells (59). A reduced dependence on costimulatory molecules and the ability to respond to lower levels of antigen than naïve cells (60) are other qualitative enhancements contributing to the memory phenomenon.

The molecular mechanisms supporting these qualitative cellular changes are not yet completely clear. Farber and colleagues separated memory from naïve cells on the basis of CD45 isoform expression and examined intracellular signaling events following restimulation of the different cell populations. They found that stimulation of naïve cells leads to higher levels of tyrosine-phosphorylation than in memory cells and also that memory cells do not phosphorylate ZAP-70, though this molecule could still interact with the CD3/TCR complex. Phosphorylation levels of ZAP-70-related kinase and p72syk were unchanged by the memory status of the cells (61).

Another group has reported that immunological synapse formation occurs more rapidly in memory than naïve cells and also that some lipid-raft microdomains present on the surface of memory but not naïve cells contain high densities of signaling molecules (62). Quicker synapse formation and entry into the cell cycle following antigen exposure by memory cells compared to naïve cells, as well as their shorter intermitotic period (63), predicts that some lasting cytoskeletal differences result from antigen experience. Alpha-adducin, a cytoskeletal regulatory protein, is phosphorylated following TCR stimulation and its expression subsequently downregulated (64). Expression in resting memory cells remains lower than in naïve cells. Though not yet proven, α-adducin may function in a maintaining more rigid cytoskeleton in naïve cells.

Qualitative changes in the ability of memory cells to respond to antigen are now evident. These results, interpreted without knowledge of cell specificity, have led many to wrongly conclude that an increase in antigen-specific cell frequency is a component of CD4+ T cell memory.

Focus of Dissertation Research and Experimental Strategy

I have used the DO11.10 adoptive transfer model system (46) to quantify the possible contribution of an increase in antigen-specific cell frequency to CD4+ T cell memory. In this system, TCR transgenic CD4+ T cells that recognize $OVAp_{323-339}$ are transferred from transgenic mice into normal BALB/c recipient mice. Transgenic, antigen-specific cells can be identified with the monoclonal antibody KJ1.26, which recognizes an epitope on the transgenic TCR. Adoptive transfer recipient (ATR) mice were typically immunized one to two days following cell transfer. Mice were sacrificed at various time points following immunization and transgenic population sizes measured by flow cytometry or immunohistochemistry. Activation/memory marker expression by antigen-specific cells was measured by flow cytometry. The functional responses of naïve and memory cells were compared on the basis of IL-2 and IFN-γ expression following *in vitro* restimulation with OVAp. This information, in combination with the knowledge of the exact number of antigen-specific cells present, permitted comparison of the relative contribution of increased antigen-specific cell frequency to enhanced functional response. The expansion and contraction dynamics of the antigen-specific population were confirmed in another CD4+ TCR transgenic system, the OT-II system, and also compared to CD8+ dynamics using the OT-I system.

ENHANCED RESPONSIVENESS TO ANTIGEN CONTRIBUTES MORE TO IMMUNOLOGICAL MEMORY IN CD4 T CELLS THAN INCREASES IN THE NUMBER OF CELLS

by

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Abstract

Although immunological memory is characterized by both an increase in the frequency of antigen-specific T cells and a qualitative change in the pattern of their subsequent response, it is not clear which of these components is more significant in the overall enhanced response to secondary stimulation. To address this question for the CD4+ T cell response, TCR Tg T cells were adoptively transferred to normal syngeneic mice that were immunized with the relevant peptide. After the initial expansion of TCR Tg T cells, the size of the subsequent memory population of T cells was approximately the same as the size of the starting population, independent of the number of TCR Tg cells initially transferred. This result was not due to redistribution of memory cells into non-lymphoid tissues, although the relative frequency of antigen-specific T cells in these sites was increased after immunization. The fraction of the antigen-specific TCR Tg cells that responded by production of either IL-2 or IFN-γ *in vitro* was substantially higher after immunization. Thus, the increased frequency of functionally responsive T cells was primarily due to a higher fraction of responding T cells, rather than a substantial increase in the absolute number of antigen-specific CD4+ TCR Tg T cells.

Introduction

Understanding the mechanisms operative in the development of T-cell memory is a central goal in the development of effective vaccination strategies for both infectious diseases and other clinically relevant immune responses. A key measure of an effective immunization is the demonstration of an increased frequency of antigen-specific T cells in the population, following the conceptual scheme of the clonal selection theme first ar-

ticulated almost 50 years ago (1). The clonal selection theory combines two simple ideas — that individual lymphocytes possess a single antigenic specificity and that changes in the frequency of such cells within a population are driven by antigen — and provides a symmetrical mechanism that accounts for both memory by an increased number of cells and tolerance by a decreased number of cells. Despite many refinements in our understanding of lymphocyte biology, this concept remains a central feature of our approach to experimentally measuring the effectiveness of a particular immunization. The conventional approach was to measure the precursor frequency of cells that could mediate a particular functional activity by limiting dilution analysis and assume that this frequency corresponded to the physical number of antigen-specific cells (2-6). More recently, a common surrogate for T cell memory is the frequency of T cells that produce cytokines immediately after *in vitro* antigen stimulation. This measure obviates the requirement for substantial growth of the precursor cells in order to experimentally detect the functional response. Both of these approaches make the fundamental assumption that all of the antigen-specific cells functionally respond when exposed to antigen in the particular assay system used.

The advent of tetramers of MHC molecules with a specific peptide was a significant advancement that allows the direct physical measurement of cells that bind a particular peptide/MHC (pMHC) epitope, independent of the functional capacity of these cells (7,8). Due to the greater stability of pMHC class I tetramers, most of the systems analyzed with this approach have been CD8 T cell responses (9-11), although some pMHCII tetramers have been developed more recently (12). However, analysis of CD4 T cell responses by using an alternative approach focusing on TCR Tg T cells has demonstrated

substantial heterogeneity in the functional cytokine responses, even within a population of T cells with exactly the same clonal TCR sequence (13,14). Similar results have been found with influenza-specific clones of CD4+ T cells obtained from the periphery of normal mice (15). While pMHC tetramer binding allows the analysis of normal (non-transgenic) T cells, the avidity threshold for tetramer binding is not necessarily the same as that required for a particular functional response.

Another issue that complicates the rigorous measurement of the number of memory cells present in an organism is the altered recirculation pathways of memory cells compared to naive cells (16-18). T cell activation leads to altered chemokine receptor expression and differential trafficking of naïve, effector, and memory cells through various tissue compartments (19). The situation is further complicated by the existence of effector and central memory T cells that have distinct recirculation patterns (20). The relative enrichment of both CD4 (21) and CD8 (22) memory cells in non-lymphoid tissues has been well documented, but this analysis has not been coupled with a precise measurement of the absolute number of T cells present in naïve and memory situations. Thus the question remains: To what degree does an increase in the frequency of a single clonotype of antigen-specific $CD4^+$ T cell contribute to immunological memory?

To address this question, we have employed the DO11.10 adoptive transfer system (23) to measure the expansion and contraction of $CD4^+$ T cells following immunization of the ATR mice. In this system, antigen-specific TCR Tg cells are detected with the KJ1.26 monoclonal antibody KJ1.26, which recognizes the DO11.10 OVAp-specific, Tg TCR (24). Using a combination of flow cytometry and immunohistochemistry, we have measured the number of T cells present in ATR mice both immediately before, and 30

days following, immunization. Our data show that the fraction of KJ1.26+ TCR Tg cells that expressed cytokines after peptide restimulation was substantially higher within the memory pool, suggesting that an altered quality of response is a more critical feature of memory among CD4 T cells than a physical increase in numbers of antigen-specific cells.

Materials and methods

Mice

The DO11.10 Tg TCR mice were the kind gift of Dr. Dennis Loh. DO11.10 mice and BABL/cByJ mice (the Jackson Laboratory, Bar Harbor, ME) were bred in our facility in accordance with NIH regulations.

Flow cytometry

Cell-surface staining was performed using standard procedures. KJ1.26 mAb was purified and conjugated to fluorescein isothiocyanate (FITC) by Southern Biotechnology Associates (Birmingham, AL). KJ1.26^{PE} and F4/80^{PE} were purchased from Caltag (Burlingame, CA). All other antibodies were purchased from BD-Pharmingen (San Diego, CA). Splenocytes or lymph node cells were incubated with KJ1.26^{FITC} specific for the transgenic TCR clonotype and PE-Cy5 conjugated anti-CD4 (RM4-5). For large list-mode acquisitions, only F4/80 negative cells were analyzed to exclude CD4^{lo} non-T cells and autofluorescent cells for a precise enumeration of very rare CD4+KJ1.26+ T cells. For analysis of activation and memory marker expression, cells were incubated with KJ1.26^{FITC}, CD4^{APC}, F4/80^{PE}, and biotin-conjugated α-CD25 (7D4), α-CD45RB (16A), α-CD62L (MEL-14), or α-CD122 (TM-β1). Biotin-labeled primary mAbs were devel-

oped with streptavidin- conjugated Red^{670} (Invitrogen, Carlsbad, CA). Flow cytometry was performed on either a Becton Dickinson FACScan or FACScalibur and analyzed with CellQuest software on list-mode acquisitions of up to 1.5 x 10^6 lymphocytes based on forward and side scatter.

Adoptive transfer and immunization

Pooled DO11.10 splenocytes and lymph node cells were analyzed by flow cytometry to determine the percentage of $CD4^+KJ1.26^+$ cells in the population. An appropriate number of cells sufficient to achieve 0.04 x 10^6, 1 x 10^6, or 25 x 10^6 were injected into the tail veins of recipient mice in a final volume of 0.5 ml PBS. For analysis of cell division, 5 x 10^6 cells per ml were incubated in 5 μM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes, Eugene, OR), for 8 min at 37°C. Labeling was quenched by the addition of newborn calf serum, and the cells were washed twice in serum-free PBS before transfer into recipient mice. One to three days following cell transfer, recipient mice were sacrificed or immunized with 100 μg $OVA_{323-339}$ in 100 μl of a multiple emulsion adjuvant (Pluronic F-127, squalene, Span 80, Tween 80, and Triton X-100 purchased from Sigma (St. Louis, MO)) adapted from Tomasi *et al.* (25). When compared with CFA, multiple emulsion adjuvant adjuvant generated higher responses of DO11.10 $CD4^+$ T cells as measured by both induction of IL-2 expression in the primary response and expansion of antigen-specific cell numbers (unpublished observations). Fifty μl of adjuvant was injected i.p., 25 μl s.c. at the base of the tail, and 25 μl s.c. at the scruff of the neck.

Quantification of antigen-specific T cells

Mice were sacrificed without immunization (day 0) or 5, 10, or 30 days after immunization. On the day of sacrifice, lymph nodes and spleens were harvested from the recipient mice. Half of each compartment was placed in an optimal cutting temperatureT block and snap frozen in liquid nitrogen. The remaining half was teased into suspension and counted in a hemocytometer. Cells were analyzed by flow cytometry to determine the percentage of $CD4^+$ and KJ^+ cells present. For immunohistochemistry, four micron-thick sections were cut from the frozen tissue blocks and immediately dried in acetone. Sections were next incubated in FITC-labeled KJ1.26 at room temperature and subsequently with horseradish peroxidase conjugated anti-FITC antibody (Vector Labs, Burlingame, CA). For anti-$CD4^+$ staining, cells were incubated in purified anti-CD4 antibody (H129.19 from BD Pharmingen), followed by a biotin-labeled anti-rat IgG antibody and ABC (Dako, Carpinteria, CA). Bound antibody was detected by precipitation of 3,3'-diaminobenzidine (Dako), a substrate of horseradish peroxidase. Sections were counterstained with methyl green, and positive cells were quantified with bright field microscopy and direct counting. Tissue area was measured by grid point counting.

To measure the number of $KJ1.26^+$ cells present in non-lymphoid compartments, each organ was weighed at the time of sacrifice, and the number of $KJ1.26^+$ cells per mm^2 of tissue (4-μm-thick sections) was calculated in a given mass of tissue assuming a density of 1 gram per ml. For the lamina propria, the length of the small intestine and the contribution of the lamina propria to the cross-sectional area of the small intestine were measured. After determining these two values, the number of $KJ1.26^+$ cells present in the entire lamina propria was calculated using the same technique as used for the non-

lymphoid compartments. The number of KJ1.26+ cells present in Peyer's Patches was calculated by finding the ratio of KJ1.26+ cells to all CD4+ cells by immunohistochemistry, and the number of CD4+ cells per average Peyer's patch was measured by flow cytometry. Observations were made to detect KJ1.26+ populations in several other tissues, including salivary gland, kidney, heart, and smooth muscle. Extremely rare positive events were present in these tissues, but we calculated the combined populations of these compartments to be less than 300 cells per mouse and thus not a significant contribution to the whole body population of over 2×10^5 KJ1.26+ cells.

Detection of cytokine-producing cells in vitro

Splenocytes from adoptive transfer recipient mice that received 4×10^6 CD4+KJ1.26+ cells were plated at 3×10^6 cells per well in a 48-well plate and stimulated for 5 hr in RPMI-1640 complete media (10% FCS). GolgiPlug was added to the wells for the final 2.5 hr of stimulation. Cells were then stained with KJ1.26^{PE} (Caltag) and a cocktail of biotinylated antibodies, F4/80 (Caltag), PK136, 1D3, and SF1-1.1 (BD-Pharmingen) to exclude macrophages, NK cells, B cells, and all MHCII-expressing cells, respectively. Biotinylated antibodies were developed with streptavidin-conjugated Red^{670} (Invitrogen), and CD4-expressing cells were detected with APC-conjugated RM4-5 (BD-Pharmingen). Cytokine staining was performed with JES6-5H4^{FITC} to detect IL-2-producing cells and XMG1.2^{FITC} to detect IFNγ-producing cells using the Cytofix-Cytoperm system from BD-Pharmingen, according to the manufacturer's instructions.

Results

In vivo expansion and contraction of antigen-specific CD4+ T cells

To determine the effect of initial antigen-specific cell frequency on T-cell population dynamics following exposure to antigen, various numbers of $CD4^+KJ1.26^+$ T cells from DO11.10 donor mice were adoptively transferred into normal BALB/c recipient mice. Immunization with OVAp triggered clonal expansion in all recipients. In each case, the antigen-specific populations present in the lymph nodes (Fig. 1a) and spleen (Fig. 1b) expanded by day 5 and then began a contraction phase until the last time point at day 30. For each cell dose, the number of antigen-specific cells remaining on day 30 was approximately the same as the starting population (Fig. 1a and 1b). However, a striking inverse relationship exists between the initial cell dose and the extent of cellular expansion. The 25 x 10^6 cell dose expanded only 2-5 fold, while the 0.04 x 10^6 and 1 x 10^6 cell doses expanded by about 40- and 70- fold, respectively. Likewise, the expansion and contraction of each dose of KJ1.26+ cells was observed in histologically stained lymph nodes, spleen, and multiple other tissues (Fig. 2). $CD4^+KJ1.26^+$ cells did not expand in recipients after immunization with KLH (data not shown).

To further characterize the *in vivo* expansion of the KJ^+ populations following immunization with OVAp, CFSE-labeled DO11.10 cells were transferred into normal BALB/c mice. In agreement with the increases in absolute cell numbers, smaller starting populations of $CD4^+KJ1.26^+$ cells underwent more cell cycles of division than larger starting populations by day 3 (Fig. 3). Analysis of CSFE dilution was also performed at day 2 and day 5 and showed similar results, but by day 5 the dilution of CSFE were indistinguishable from autofluorescence, thereby obscuring the differences between the

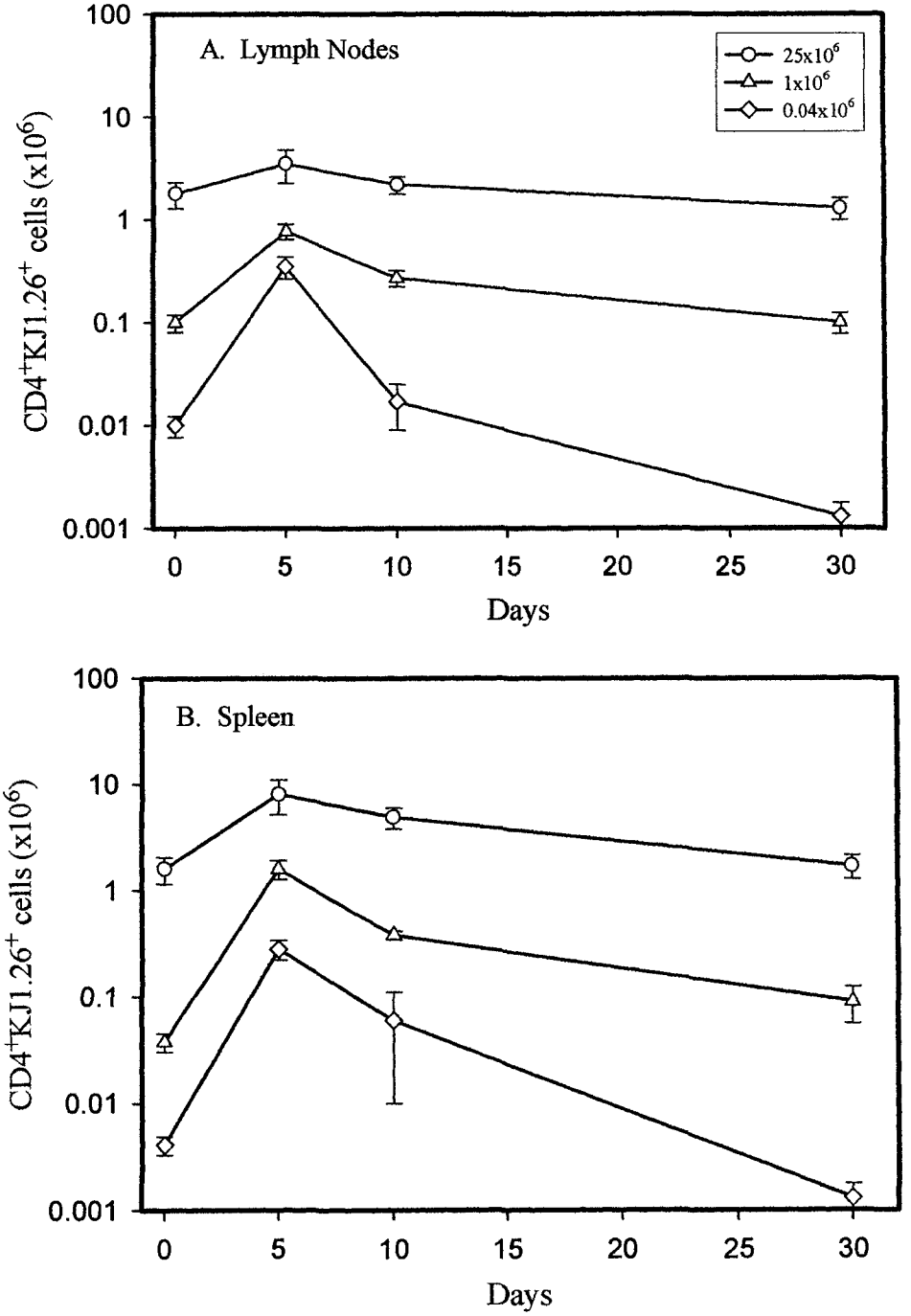

Figure 1. Expansion and contraction of adoptively transferred CD4+KJ1.26+ cell populations in recipient BALB/c ByJ mice as measured by flow cytometry. Cell transfers were performed on day -2, and mice were sacrificed on day 0 (unimmunized) and on days 5, 10, and 30 following immunization with 100 μg of OVA peptide in adjuvant. Circles–25 x 10^6 cell dose; triangles–1 x 10^6; diamonds–0.04 x 10^6. Error bars indicate the standard error of the mean. (A.) Lymph nodes. (B.) Spleen.

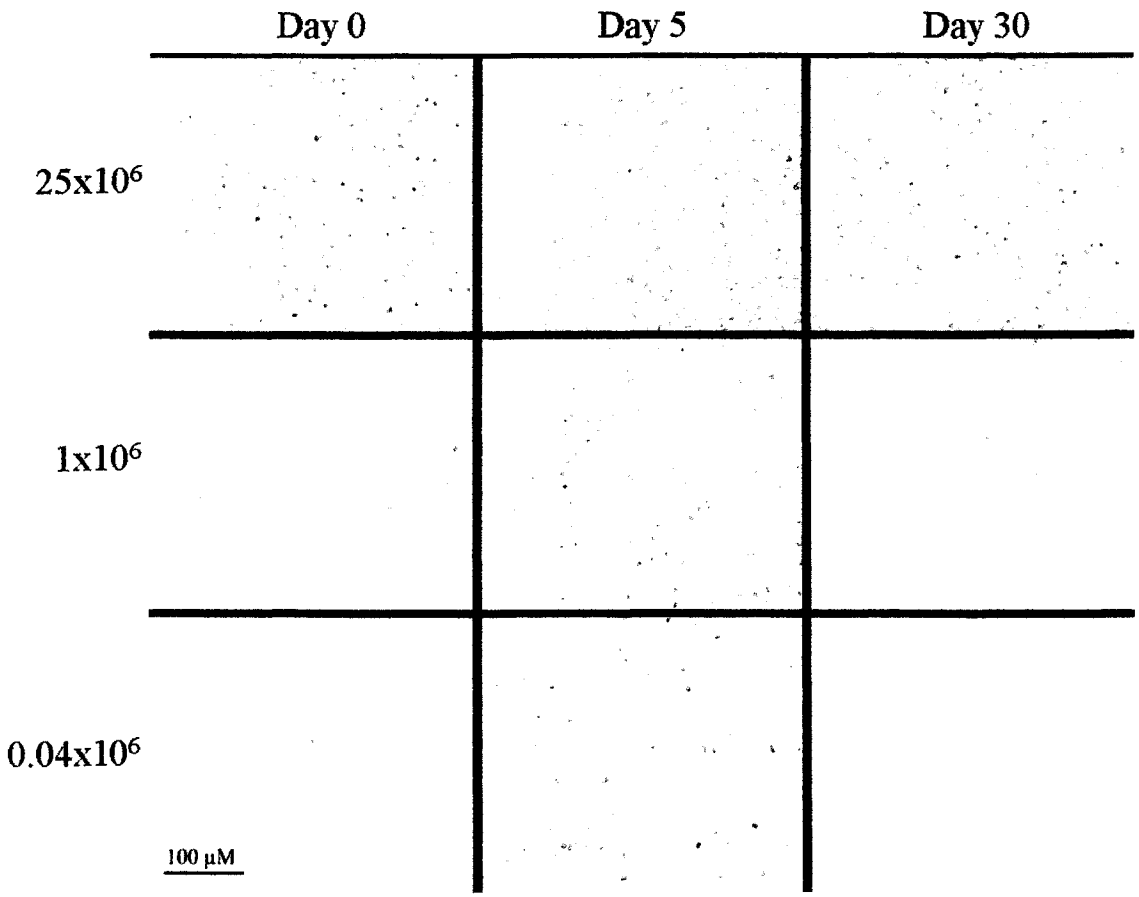

Figure 2. Photomicrograph of lymph node sections stained with KJ1.26 on days 0, 5, and 30 following immunization. Mice received 0.04 x 10^6, 1 x 10^6, or 25 x 10^6 CD4+KJ1.26+ cells. Equivalent expansion and contraction of the numbers of KJ1.26+ cells per square millimeter was observed in the spleen.

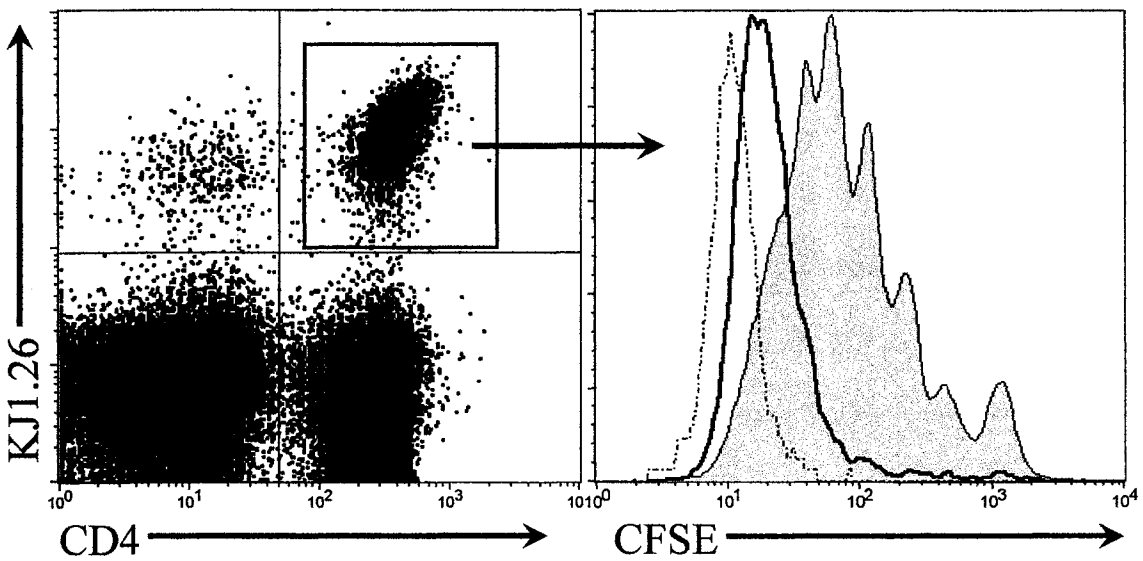

Figure 3. CFSE dilution profiles of CD4+KJ1.26+ lymphocytes from the spleen on day 3 following immunization. 25 x 10^6 – grey fill; 1 x 10^6 – solid line; 0.04 x 10^6 – dotted line.

different initial cell doses (data not shown). $CD4^+KJ1.26^+$ cells from unimmunized control mice did not proliferate (data not shown).

Activation and memory marker expression by KJ^+ cells

Decreased expressions of CD45RB and of CD62L are commonly used to distinguish antigen experienced from naïve cells. Expression of four activation/memory markers by memory and control immunized cells was examined on day 30 (Fig. 4). Expression of CD25, the IL-2Rα subunit, was up-regulated on day 1 after immunization (data not shown), but returned to control levels by day 30. Expression of CD122, the IL-2Rβ subunit, was also increased soon after immunization (data not shown) but was expressed at elevated levels through day 30 compared to cells from mice immunized with control antigen. As expected, $KJ1.26^+$ cells from mice immunized with OVAp showed reduced expression of CD45RB and CD62L. Similar results were found in spleen cells.

Antigen-specific $CD4^+$ T cells in non-lymphoid tissues

The contraction of activated antigen-specific cell populations results mostly from activation-induced cell death (26), but redistribution into other compartments may contribute to the disappearance of TCR Tg cells from the spleen and lymph nodes. To quantify redistribution into other compartments 30 days after immunization, we used an immunohistochemistry technique (Fig. 5) with accuracy equal to flow cytometry (27). The number of KJ^+ cells present in the lamina propria of the small intestine, Peyer's Patches, lung, liver, salivary gland, heart, kidney, and skeletal muscle was measured on days 0 and 30 in mice that received 1×10^6 $KJ1.26^+$ cells (Fig. 6a). Although the fraction of the total

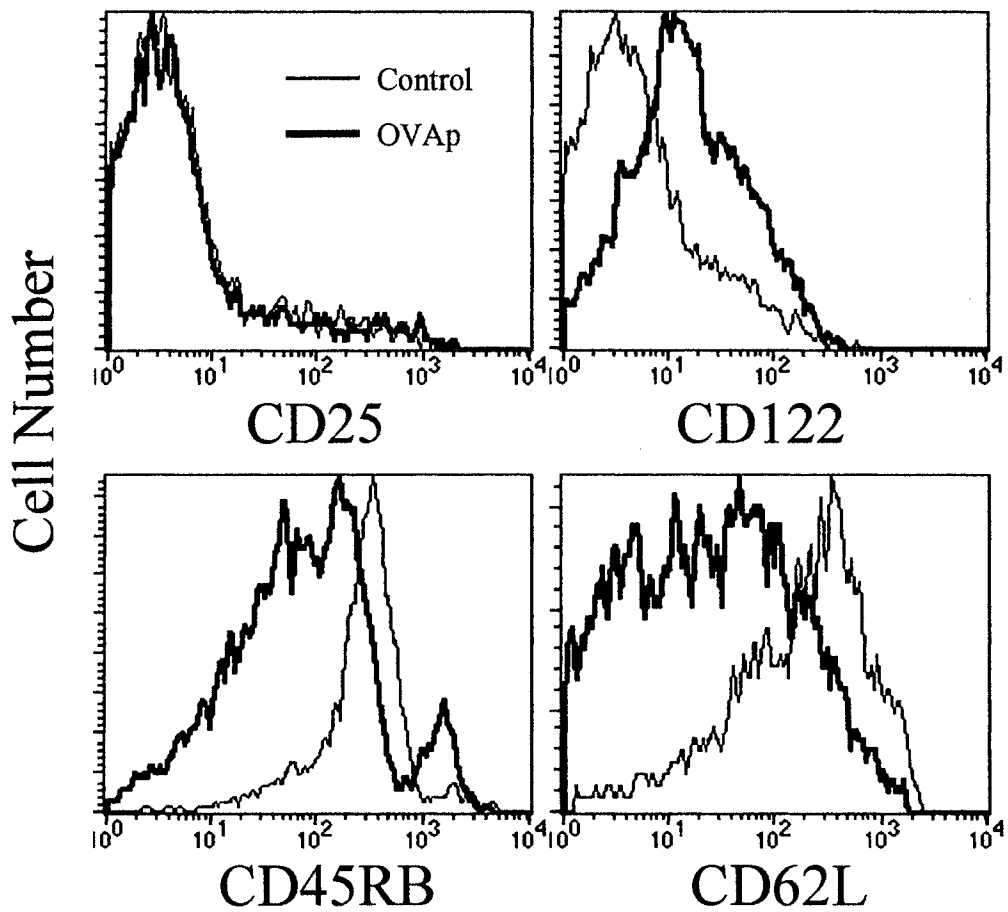

Figure 4. Comparison of activation and memory marker expression by CD4+KJ1.26+ lymph node cells from OVAp immunized (thick line) and KLH control immunized (thin line) mice 30 days following immunization. Modulation of activation and memory marker expression by CD4+KJ1.26+ splenocytes was equivalent.

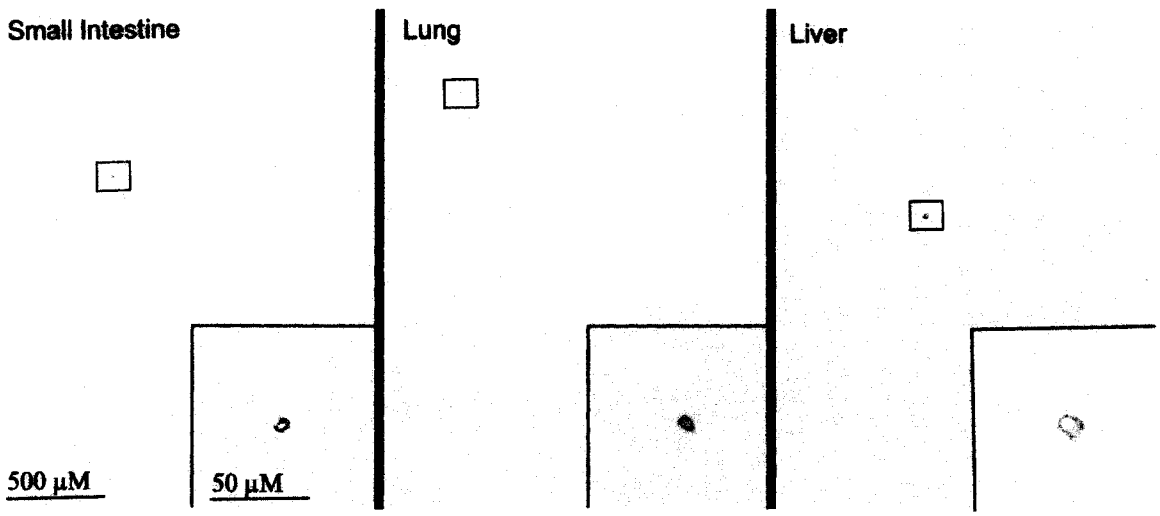

Figure 5. Photomicrograph showing KJ1.26+ cells in the small intestine, lung, and liver in mice which received 1 x 10^6 CD4+KJ1.26+ cells.

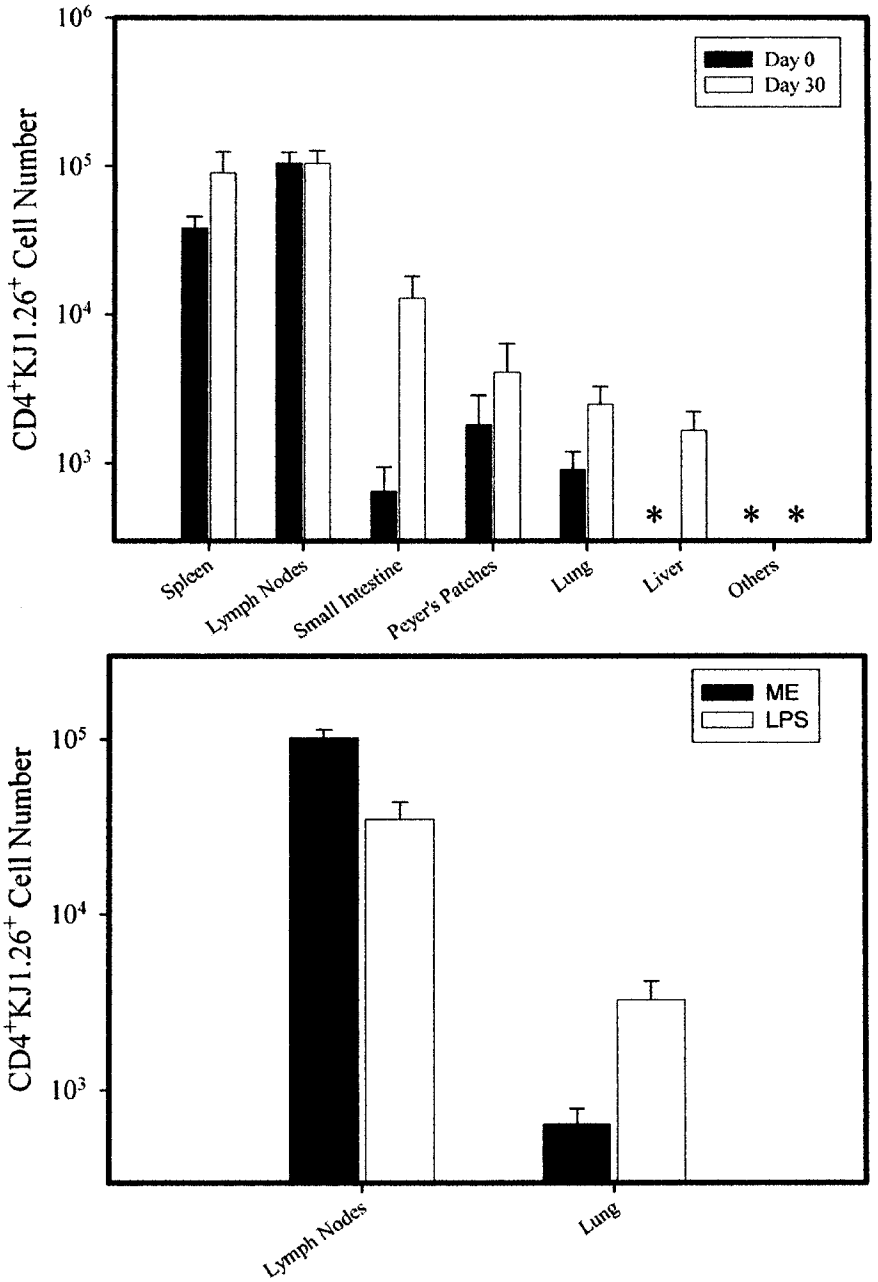

Figure 6. Absolute numbers of KJ1.26+ cells in lymphoid and non-lymphoid compartments. (a.) KJ1.26+ cell numbers in spleen, lymph nodes, small intestine, Peyer's patches, lung, liver, and combined other compartments (salivary gland, kidney, heart, and skeletal muscle) on day 0 without immunization (black) and day 30 following immunization (unfilled). Asterisks indicate total population sizes below the limit of detection for the respective compartment. Results are from four mice on day 0 and five mice on day 30. Adoptive transfer mice received $1x10^6$ CD4+KJ1.26+ cells. (b.) KJ1.26+ cell number in the lymph nodes and lungs of adoptive transfer recipient mice mice that were immunized with OVAp in multiple emulsion adjuvant or with OVAp in LPS. Results are from three mice for each condition.

CD4 T cells that co-expressed KJ1.26 was significantly increased in the non-lymphoid compartments (Fig. 6a), the vast majority of KJ1.26$^+$ cells present in the mouse resided in the lymphoid compartment.

This result contrasts with that reported by Reinhardt *et al.* which showed a net redistribution of antigen-specific T cells from the lymph nodes to the non-lymphoid tissues 30 days following immunization with OVAp and LPS. Thus, we compared the effect of immunization route and adjuvant on antigen-specific cell distribution 30 days after immunization in adoptive transfer recipient mice, which received 3 x 10^6 CD4+KJ1.26+ cells (Fig. 6b). These results showed that i.v. immunization using LPS as an adjuvant resulted in only one-third as many CD4+KJ1.26+ T cells remaining in the lymph nodes compartment as compared to mice that were immunized subcutaneously and intraperitoneally with multiple emulsion adjuvant. Additionally, OVAp+LPS-immunized mice showed a 5-fold increase in the number of KJ1.26+ cells found in the lungs 30 days following immunization. No significant difference was found in cell numbers in other compartments. However, the basic result, that the majority of the CD4+ TCR Tg cells in the entire animal 30 days after immunization reside in the spleen and lymph nodes, was not altered by this alternative form of antigen delivery.

Functional differences in memory and naïve cells

To determine whether the quality of the secondary response of these memory CD4+ T cells was altered, despite the minor change in total cell number, production of both IL-2 and IFN-γ after *in vitro* stimulation was measured by intracellular cytokine staining. At the time of sacrifice, 35 days after immunization, the KJ1.26+ T cells com-

prised approximately 1% of the splenic CD4+ population (Fig. 7a). Upon restimulation *in vitro*, a greater fraction of the antigen-specific cell population from adoptive transfer recipient mice previously immunized with OVAp produced IL-2 and IFNγ than did populations containing similar frequencies of naïve DO11.10 cells at all doses of antigen tested (Fig. 7b). Approximately 30% of the memory $CD4^+KJ1.26^+$ population produced IL-2, compared with only 10% of the naïve $CD4^+KJ1.26^+$ population after stimulation with 30 μg/ml OVAp (Fig. 8a). The fraction of antigen-specific cells producing IFNγ at this antigen dose (11% positive) was also substantially higher in the memory population, compared to 0.9% positive in the naïve population (Fig. 8b). Thus, the number of $OVA_{323-339}$-responsive cells was substantially increased within the memory population, but this increase was due to a higher fraction of the clonotype-positive cells making a functional response, not an increase in the actual number of antigen-specific cells. Although immunization may have activated some non-TCR Tg cells in these adoptively transferred mice, about 90% of the total cytokine-expressing cells measured by intracellular staining were KJ1.26+, indicating that the TCR Tg population comprises the bulk of the $OVA_{323-339}$ peptide response in this experimental model (data not shown). A small fraction of the KJ1.26+ cells expressed CD8 rather than CD4, and they produced IFNγ at higher levels than the CD4+ cells. Selection of TCR Tg T that recognize their specific antigen across MHC restriction barriers and thus develop as either CD4+ or CD8+ T cells has been previously reported (28). The increased fractional response of antigen-specific CD4+ T cells contributes more to the heightened memory response than does the increase in numbers of antigen-specific cells (Fig. 8c).

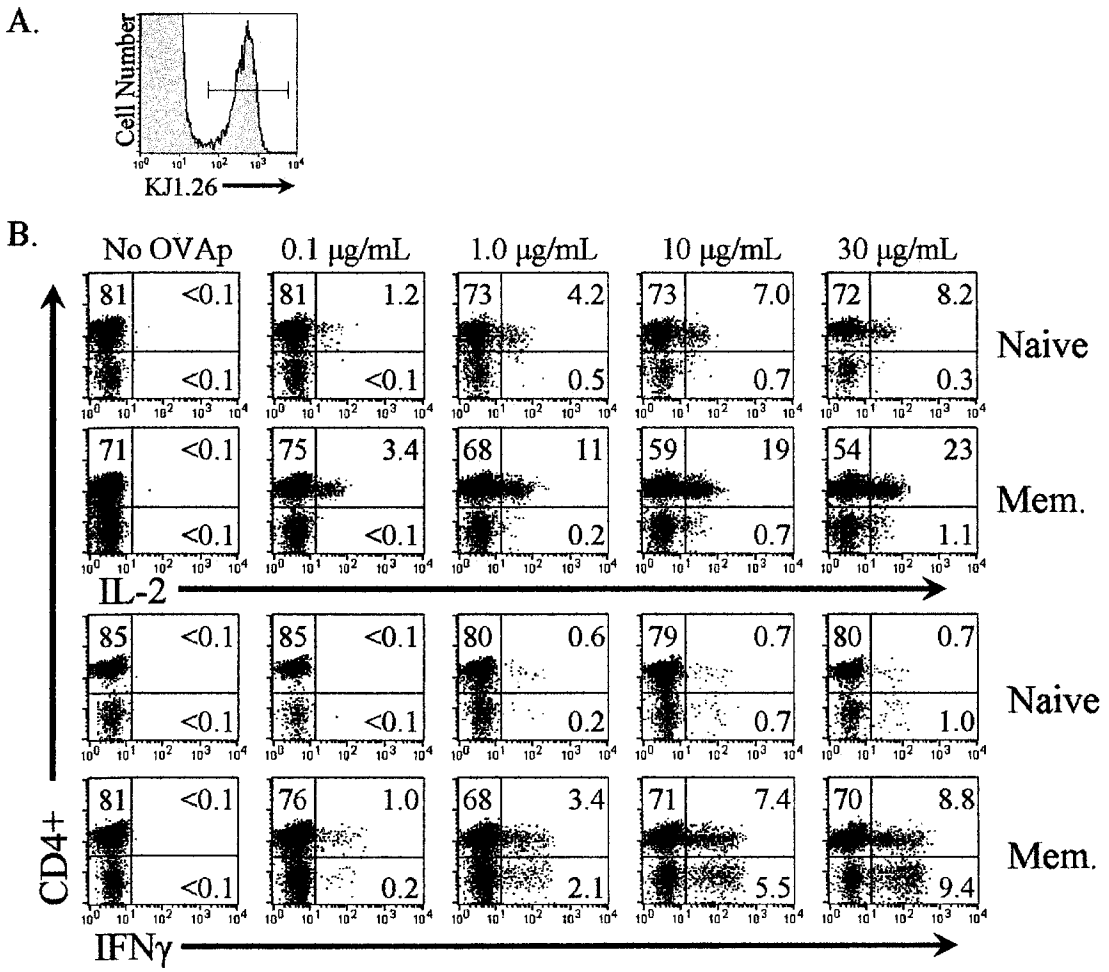

Figure 7. Enhanced functional response in memory cells compared to naïve cells in adoptive transfer recipient mice that received $4x10^6$ CD4+KJ1.26+ cells. (a.) KJ1.26+ cells composed approximately 1% of the total splenic CD4+ lymphocyte population. (b.) Intracellular IFNγ and IL-2 production by KJ+CD4+ and KJ+CD4- splenocytes from OVAp-immunized adoptive transfer recipient mice and unimmunized control mice following a 5hr *in vitro* stimulation. Plots are gated on the KJ1.26+ population.

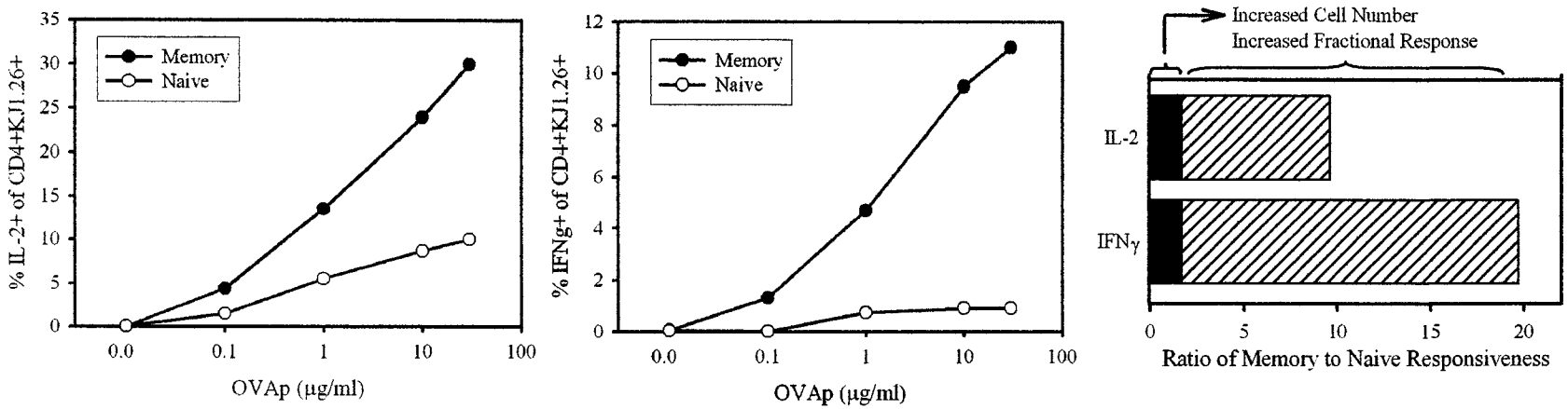

Figure 8. The percentage of CD4+KJ1.26+ cells which produce IL-2 (A.) and IFNγ (B.) following *in vitro* stimulation with OVAp is greater in memory populations (black fill) than in populations of naïve cells (unfilled). (C.) The Relative contributions of ncreased cell number and increased fractional response to the ration of memory to naïve responsiveness.

Discussion

An increase in the number of antigen-specific cells has conventionally been regarded as a key component of the increased intensity of secondary immune responses. This measure has been widely applied in vaccine studies, in which the number of cells capable of responding to a specific antigen as judged by a given assay during a recall challenge was measured rather than physical identification of cells with identical TCR sequences. The relative contribution of increased precursor frequency compared to the enhanced fractional response within a clonotype is unknown. Our results indicate that net increases in the absolute number of antigen-specific CD4+ T cells is relatively modest compared to the more substantial enhanced fractional response and dose sensitivity which are characteristic of memory populations.

Our data show that, regardless of the initial size of the adoptively transferred cell population, the respective populations at 0 and 30 days in the lymphoid compartments are approximately the same size. Kearney *et al.* saw similar expansion and contraction when they first transferred a single dose of CD4+ TCR Tg cells into recipient mice (23). When the adoptive transfer results in a supra-physiological frequency, the final population size might be determined by a limitation in the total size of a particular specificity. However, the result that the final population of memory cells is roughly equivalent to the stating cell number over a side range of initial frequencies suggests that some property inherent to the individual T cell governs the final population size. The contraction of antigen-stimulated CD4+ populations to approximately the same population size as before antigenic stimulation contrasts with the well-demonstrated biology of the expansion of CD8+

cells, which expand and subsequently contract to a population size that may be many fold larger than the naïve population (29-31).

Interestingly, the behavior of activated cells is affected more by total population size than by the behavior of resting cells. During the several days following immunization, the adoptive transfer cell populations proliferate in response to antigen regardless of their initial population size, but the highest transferred cell dose expanded approximately 5-fold in the spleen and 2-fold in the lymph nodes, while the 0.04×10^6 transfer population expanded 73-fold in the spleen and 37-fold in the lymph nodes. The blunted response in mice that received 25×10^6 $CD4^+KJ1.26^+$ cells likely results from the high frequency of antigen-specific cells and the consequent competition for limited resources necessary for antigen-specific population expansion. These results support previously published findings showing that labeled TCR Tg populations transferred into TCR Tg recipient mice compete for resources available in the host animal (32) and that intraclonal competition can suppress the number of antigen-specific cells that produce IL-2 following immunization (33). Additionally, we noted that the magnitude of change in activation marker expression by the largest transfer cell dose was also less than the change in activation marker expression shown by the smaller transfer doses (data not shown).

Another novel observation reported here concerns the pattern of recirculation of the memory T cells between lymphoid and non-lymphoid tissues. Although the fraction of KJ1.26+ OVAp-specific CD4+ cells increased after immunization, the majority of the memory cells were found in the lymphoid tissues. Previous reports have shown conflicting results on this point. Schiemann *et al.* (31) quantified the number of antigen-responsive CD4+ and CD8+ cells in lymphoid and non-lymphoid compartments 35 days

after recall infection with Listeria and reported that the decline in the number of antigen-specific cells in the lymphoid tissues was not the result of net redistribution into non-lymphoid compartments. By contrast, Masopust *et al.*, using a VSV model system to study CD8 T cell memory, found that memory CD8 T cells preferentially localize in non-lymphoid tissues (22). However, they looked only at the percentage of the CD8+ population that was tetramer positive rather than measuring the absolute number of cells present in each compartment. Enrichment of CD8+ memory cells in these locations occurs, but this result does not indicate large-scale redistribution of bulk numbers of cells into these tissues. Reinhardt *et al.* reported a net redistribution of the antigen-specific CD4+ population into non-lymphoid compartments (21). They obtained this result by quantifying the number of TCR transgenic (OT-II) cells that express Thy1.1 present in single midline sections of an entire mouse. This single section samples many tissues simultaneously but may not accurately reflect the relative abundance of all body compartments, particularly the regional lymph nodes that are not present in a single midline section. The analysis reported here involves analysis of all body compartments with direct measurement of the mass and volume of each tissue by conventional techniques, rather than a single plane of section. Our results showed some redistribution of memory cells into non-lymphoid tissues, but we found that 93% of the antigen-specific CD4+ cells remained in secondary lymphoid tissues 30 days following immunization. It is likely that this relative enrichment of memory CD4+ cells in non-lymphoid peripheral tissues corresponds to a greater rate of recirculation through these compartments. These memory cells can gain access to the regional lymph nodes via drainage in the afferent lymph, and not merely through immigration directly from blood via the high endothelial venules (34). Precise measure-

ment of the rates at which T cells recirculate through different compartments has not been performed, but the profound T cell depletion associated with thoracic duct drainage and the short residence time of T cells in the blood (35,36) strongly suggest that these rates are quite rapid. Thus, the localization of T cells in different compartments most likely reflects a highly dynamic equilibrium of recirculation patterns rather than a static localization of T cells in these sites.

It has long been assumed that the enhanced functional response of a T-cell population after a primary response is primarily due to an increase in the frequency of the antigen-specific subset of cells. The underlying rationale for this concept is that all of the antigen specific-cells mediate functional activity, such as cytokine production, when stimulated with adequate amounts of the specific antigen *in vitro*. However, the activation of cytokine expression by individual T cells is far from uniform, even within clonal populations (13,14). Since the fraction of antigen-specific CD4 T cells that produce cytokine with optimal stimulation is actually fairly low in naïve cells, a higher functional response could be due to either an increase in the number of antigen-specific cells or to a higher probability of response for each individual cell. In the experimental system examined here, the frequency of CD4+ T cells that produce IFN-γ is increased by about 25-fold in the memory population of cells compared to the naïve cells. A relatively small proportion of this increase (about 2-fold) was due to the increase in the physical number of antigen-specific KJ1.26+ cells, while each KJ1.26+ cell was about 12 times more likely to produce this cytokine with optimal *in vitro* stimulation (Fig. 6). In addition, the memory cells show about a 10-fold increase in the sensitivity to antigen dose for functional activation. In the response to an infectious agent, this increased sensitivity to low

amounts of antigen would correlate to a greater response at an earlier time point in the growth of the infectious agent. Therefore, the increase in the quality of the response is a significantly larger component of the overall increase, although both an increase in absolute cell number and an increase in fractional response contribute to the higher functional response in the memory population.

Since the functional phenomenon of antigen-specific memory in the CD4 T cell populations can result primarily from changes in the quality of the response, the assessment of CD4 T cell function *in vitro* in various clinical conditions should also focus on the quality of the response. Measures such as a change in the pattern of cytokine expression and shifts in the sensitivity to peptide stimulation may be more relevant in some circumstances than direct frequency measurements.

Acknowledgments

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DISCUSSION

As previously shown, populations of memory CD4+KJ1.26+ T cells do not maintain expanded population sizes compared to the size of the naïve population. However, this result definitively represents only a single TCR clonotype out of all clonotypes present in the periphery. The specificities of T cells that compose the peripheral population all recognize some selecting antigen in the context of the host MHC complex. The avidities with which these cells recognize their ligands form a spectrum ranging from very low avidity to extremely high avidity recognition and affect the course of the response (65-68).

On a molecular level, avidity determines the dwell time of TCR with the loaded MHC complex (69). Very low avidity ligands fail to engage the TCR for sufficient time to trigger activation events in the cell. High avidity ligands, by contrast, have an unnecessarily long dwell time, which also has an inhibitory effect on T cell activation. Optimal cellular activation results from TCR interaction with a moderate avidity ligand.

CD4+ T cells function primarily to coordinate the immune response and they achieve this coordination in large part by secreting cytokines (70). Because cytokines are soluble and capable of diffusing away from the cell which produced them, one CD4+ T cell can affect the response of numerous surrounding cells. CD8+ T cells, by contrast, effect their response mainly through cytotoxic cell-cell contact interactions (71). One CD8+ T cell may serially kill numerous target cells, but a greater number of cells are

likely required to effect a full response compared to CD4+ T cells. Additionally, CD8+ T cells do not completely contract to the levels at which they were present prior to the immune response (72). This elevated memory CD8+ T cell frequency facilitates a more rapid secondary response, whereas the memory CD4+ T cell response in enhanced by an increase in the fraction of antigen-specific cells that respond rather than an increase in the absolute number of cells. This difference in biological function likely accounts for the distinct patterns of population expansion between the two cell types.

In models of the CD8+ T cell response, the magnitude of antigen-specific cell population expansion greatly surpasses the level of population expansion seen in CD4+ T cell model systems. In the lymphocytic choriomeningitis virus system, the virus-specific CD8+ T cell population expands from below the levels of detection prior to the response to approximately 50% CD8+ T cell population at its peak (47,73). An antigen-specific CD4+ T cell population expansion this great has never been demonstrated.

To compare the response of CD4+KJ1.26+ T cells following immunization of adoptive transfer recipient mice with another clonotype of CD4+ T cell, $CD90^{1/1}$B6 mice were transferred with 1 x 10^6 $RAG^{-/-}CD90^{2/2}$ OT-II CD4+ T cells. No anti-clonotypic antibody for the OT-II TCR was available, so adoptively transferred cells were identified on the basis of CD4 and CD90.2 expression. OT-I AT cells were identified on the basis of CD8 and CD90.2 expression. Recipient mice were immunized with 100 μg of OVAp or chicken ovalbumin peptide residues SIINFEKL on the day following cell transfer, except for day 0, mice which were sacrificed on that day without immunization. Remaining mice were sacrificed on days 5, 10, or 30 following immunization. At least three mice were sacrificed each day.

OT-II ATR mice sacrificed on day 0 had an average of 1.4×10^5 AT cells in the spleen and 1.1×10^5 AT cells in the lymph nodes. This population expanded to 5.2×10^6 in the spleen and 1.2×10^6 in the lymph nodes on day 5 following immunization. A contraction phase followed during which the AT population size was reduced to 1.9×10^5 in the spleen and 2.5×10^5 in the lymph nodes on day 10. The AT population continued to wane until day 30, when the population sizes in the spleen and lymph nodes were 7×10^4 and 6×10^4 respectively (Fig. 1). These results are similar to those generated using the DO11.10 adoptive transfer system in which the CD4+KJ1.26+ population sizes in the spleen and lymph nodes were 9×10^4 and 1×10^5 30 days following immunization. OT-II populations in unimmunized mice did not expand following immunization. 31 days after cell transfer approximately 6×10^5 and 5×10^5 of these cells were present in the spleen and lymph nodes, respectively.

In OT-I ATR cohorts, mice sacrificed on day 0 had an average of 2.4×10^5 AT cells in the spleen and 1.8×10^5 AT cells in the lymph nodes (Fig. 2). This population expanded to 2.2×10^6 in the spleen and 1.7×10^6 in the lymph nodes on day 5 following immunization. A contraction phase followed during which the AT population size was reduced to 3×10^5 in the spleen and 7×10^4 in the lymph nodes on day 10. The AT population continued to wane until day 30, when the population sizes in the spleen and lymph nodes were both 5×10^4.

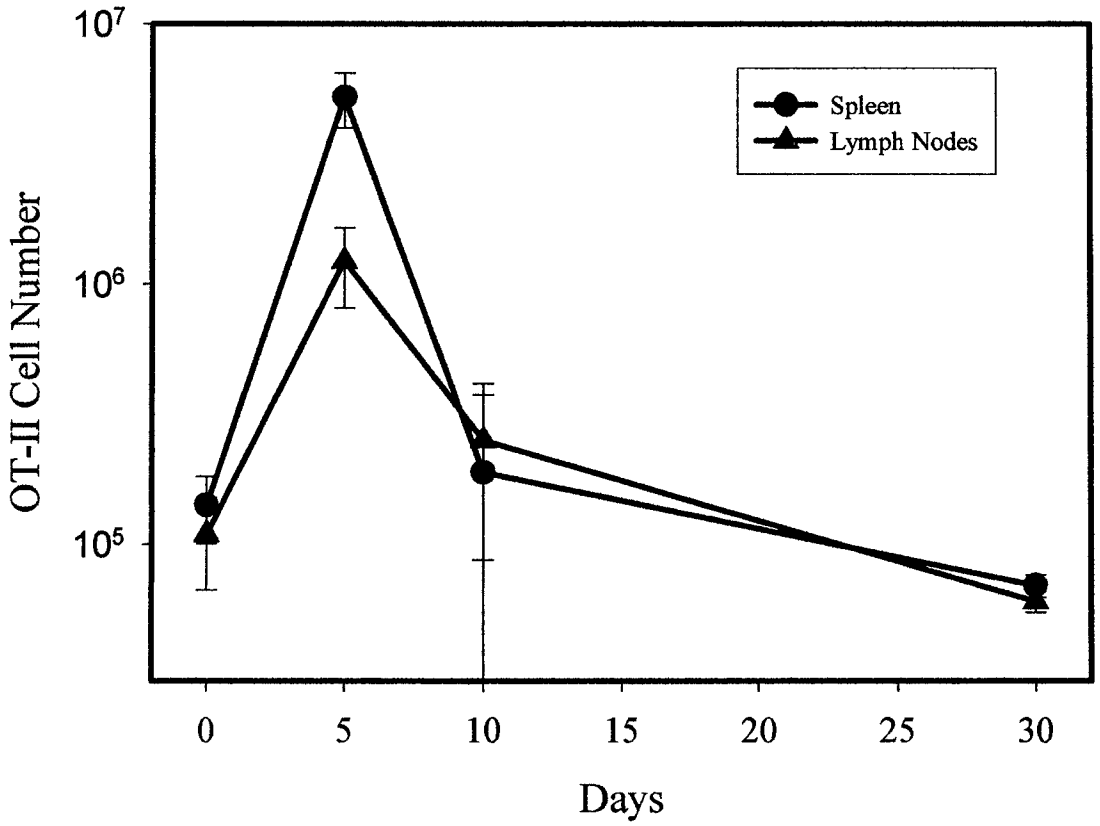

Figure 1. Expansion and contraction of adoptively transferred OT-II CD4+ T cells in the spleen and lymph nodes of recipient mice as measured by flow cytometry. Cell transfers were performed on day -2 and mice were sacrificed on day 0 (unimmunized) and on days 5, 10, and 30 following immunization with 100 μg of OVA peptide in adjuvant. Circles – spleen; triangles – lymph nodes.

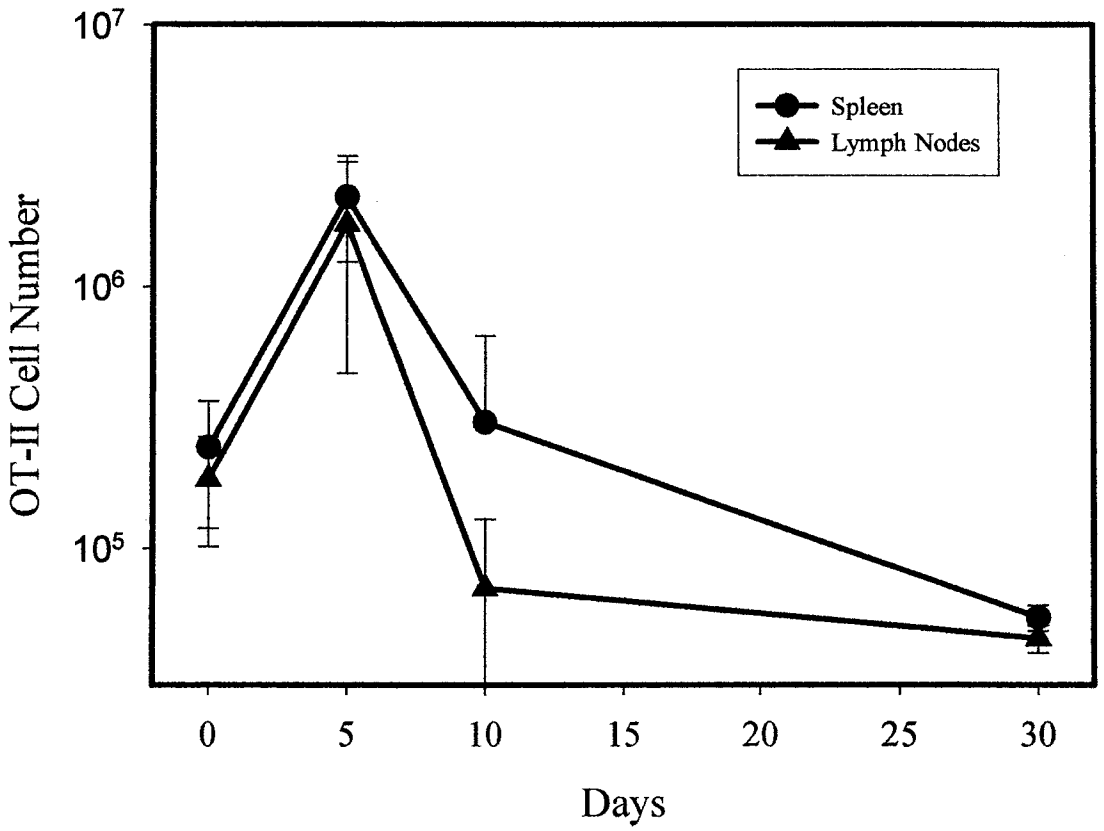

Figure 2. Expansion and contraction of adoptively transferred OT-I CD8+ T cells in the spleen and lymph nodes of recipient mice as measured by flow cytometry. Cell transfer were performed on day -2 and mice were sacrificed on day 0 (unimmunized) and on days 5, 10, and 30 following immunization with 100 μg of OVA peptide in adjuvant. Circles – spleen; triangles – lymph nodes.

SIGNIFICANCE

While observing the response of only two clonotypes of CD4+ T cells is not representative of the entire periphery, the similarity of response shown by DO11.10 and OT-II TCR Tg cells suggests that the phenomenon is not simply an artifact of the DO11.10 transgenic system. Another possible limitation, though difficult to address, is that TCR sequences which are most easily selected for cloning by *in vitro* proliferation and subsequent use in TCR transgenic line founding may represent a narrow range of avidities for their respective ligands. The approach to cloning antigen-specific TCR sequences and creating transgenic lines may have conserved a sampling bias across all TCR transgenic systems. Thus clonotypes may be present in the periphery that can recognize an antigen with sufficient avidity to generate a response but, because of the difference in avidity from TCR transgenic systems, the magnitude and manner of this response may vary.

The failure of OT-I CD8+ memory cells to persist in this adoptive transfer system contrasts with the findings of published CD8+ studies, which show a maintained expansion of the memory population following the response (47). A likely explanation for the discrepancy in results lies in the choice of model system. The experiments that have led to the current concept of CD8+ biology were largely conducted using viral infection models. Infection models create the possibility of persistence of low levels of antigen which may be extremely difficult to detect experimentally but sufficient to maintain CD8+ memory populations (74).

In contrast to soluble peptide models, infection models have the benefit of creating cell-associated antigen rather than relying strictly on the uptake of antigen by APCs for subsequent presentation to T cells. This difference may be of particular importance for CD8+ responses. Li *et al.* have compared the effect of soluble versus cell-associated antigen on CD8+ and CD4+ T cell response using the OT-I and OT-II systems (75). They report that cell-associated ovalbumin is presented 50 000-fold more efficiently to CD8+ T cells and 500-fold more efficiently to CD4+ T cells. The reduced efficiency of antigen presentation to CD8+ T cells effectively lowers the antigen dose they receive. Williams and Bevan conducted a set of experiments in which they temporally limited antigen by administering antibiotics to *Listeria* infected mice 24-48 hr following infection (76). They report that the limited infection results in the same size primary CD8+ response but a smaller subsequent population of memory cells.

The reliance entirely on soluble antigen to generate a response in the OT-I experiment reported here may account for the failure to create a sustained memory population. If this scenario proves true, it has important implications for therapeutic vaccine development against HIV, wherein a cellular immune response may be triggered but never result in the formation of effective cellular immune memory.

SUMMARY

The results communicated herein demonstrate that increased antigen-specific cell frequency is not a substantial component of CD4+ T cell memory. Analysis of the antigen responsiveness of populations of memory CD4+ T cells without the ability to physically identify antigen-specific cells have wrongly lead to the common perception that increases in antigen-specific cell number is one of the mechanisms underlying the enhanced qualitative response of memory populations.

Additionally, I have shown that over 90% of antigen-specific cells remain in the lymphoid tissues 30 days following sacrifice. Adjuvant choice was found to have a minor effect on the distribution of memory cells.

Interestingly, I was unable to demonstrate that increased antigen-specific cell frequencies are a component of CD8+ T cell memory. The failure of peptide antigen to persist *in vivo* or the failure to provide CD8+ T cells with cell-bound antigen may account for this result. Should the latter prove true, it could be of great use in the design of vaccines intended to elicit CD8+ T cell memory and presents an interesting opportunity for further research.

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Title of Dissertation CD4+ T Cell Memory and Antigen Specific Cell Frequency

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

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