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Review

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Tracking lymphocytes *in vivo*

Claire L. Adams, Natalie Kobets, Gordon R. Meiklejohn, Owain R. Millington, Angela M. Grierson, Catherine M. Rush, Karen M. Smith and Paul Garside

University of Glasgow, Western Infirmary, Glasgow, Scotland, UK

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Summary

The ability to track antigen (Ag)-specific lymphocyte populations *in vivo* has greatly increased our understanding of the location and functional status of these cells throughout the course of an immune response. Recent technical advances have enhanced researchers' capability to follow migration, activation and cellular interactions of Ag-specific lymphocytes *in situ*. It is now possible to monitor changes in T cell subsets, co-stimulatory molecules, and chemokine expression within the physiological context of secondary lymphoid organs. Furthermore, the Ag-presenting cell-T cell interaction can be studied, thus dissecting the role and timing of Ag presentation of particular dendritic cell subsets in the initiation of the immune response. The capacity to adoptively transfer small populations of Ag-specific T lymphocytes has also increased our knowledge of the physiologically important role of regulatory T cells in autoimmunity and immunosuppression. New fluorescence imaging techniques such as multicolor video microscopy, laser scanning cytometry, and multiphoton tissue imaging have provided new ways in which researchers can track cellular changes within Ag-specific lymphocytes *in vivo*. This review summarizes some of the ways in which these techniques have led to discoveries in the role of signaling cascades, cell cycle progression, and apoptosis in maintaining an Ag-specific immune response.

Key words: **adoptive transfer • Ag-specific • cellular interactions • fluorescence • lymphocytes • transgenic**

Abbreviations: **Ag** – antigen, **ALPS** – autoimmune lymphoproliferative syndrome, **APC** – antigen-presenting cell, **B7RP-1** – B7-related protein 1, **BcR** – B cell receptor, **BLC** – B lymphocyte chemoattractant, **CD** – cluster of differentiation, **CFSE** – 5,6-carboxyfluorescein diacetate succinimidyl ester, **cOVA** – chicken ovalbumin, **CTL** – cytotoxic T lymphocyte, **DC** – dendritic cell, **ELC** – EBI-1 ligand chemokine, **GFP** – green fluorescent protein, **HEL** – hen egg lysozyme, **ICOS** – inducible costimulator, **IFN** – interferon, **IL** – interleukin, **LSC** – laser scanning cytometer, **MHC** – major histocompatibility complex, **PI3K** – phosphoinositide 3-kinase, **SLC** – secondary lymphoid tissue chemokine, **SLE** – systemic lupus erythematosus, **SMAC** – supramolecular activation cluster, **TcR** – T cell receptor, **Tg** – transgenic, **TUNEL** – terminal deoxynucleotidyl-transferase dUTP nick end labeling.

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Author's address: Prof. Paul Garside, Division of Immunology, Infection and Inflammation, University of Glasgow, Western Infirmary, Glasgow, Scotland, UK, G11 6NT, tel.: +44 141 211 2153, fax: +44 141 337 3217, e-mail: P.Garside@clinmed.gla.ac.uk

INTRODUCTION

With the development of antigen (Ag)-specific T cell receptor (TcR) and B cell receptor (BcR) transgenic (Tg) mice, it is now feasible to track single Ag-specific lymphocyte populations throughout the course of an immune response. Adoptive transfer of the Tg cell populations into naïve recipients with the same genetic background increases the precursor frequency of Ag-specific lymphocytes sufficiently to allow researchers to locate and functionally assess these cells in the physiological context of a normal animal, early after Ag exposure[36, 69, 113]. Prior to the availability of such Tg mice, it was extremely difficult to track lymphocytes *in vivo* during the early phases of an immune response, i.e. before significant clonal expansion, due to the low precursor frequency of Ag--specific B and T cells.

In our laboratory, the ovalbumin-specific DO11.10 TcR Tg mouse is used as a donor to produce the naïve traceable T cell population[125, 133–135]. The $CD4^+$ T cells of the DO11.10 TcR Tg mice are specific for a chicken ovalbumin (cOVA) peptide (amino acid residues 323-339) in the context of the major histocompatibility complex (MHC) class II molecule $I\text{-}A^d$ [48] and can be identified using the clonotypic monoclonal antibody KJ1-26[103]. The ability to track Ag--specific T cell populations using techniques such as immunohistochemistry and flow cytometry has led us and several other groups to study a range of immune responses *in vivo*, including the activation and development of T cell phenotypes following the induction of immunity, autoimmunity, allergy, and tolerance and the trafficking of effector lymphocyte populations[16, 56, 62, 72, 97, 114].

Furthermore, the availability of BcR Tg mice has enabled Ag-specific B and T cell interactions to be examined at the early stages of the germinal center formation at the start of the humoral immune response. Routinely, we use MD4 BcR Tg mice, which are specific for hen egg lysozyme (HEL)[39], and can be identified using an antibody specific for the IgM^a allotype. As the TcR Tg and BcR Tg lymphocytes used recognize different Ags, it is necessary to chemically couple these proteins to facilitate cognate interactions between the T and B cells[36]. HEL and cOVA are chemically linked to allow HEL-presentation from the DO11.10 Tg T cells (cOVA-specific) to the MD4 Tg B cells (HEL-specific) at the early stages of germinal center formation.

This review will be separated into two parts. First, an evaluation of a selection of some of the advances in immunology resulting from the enhanced capability to track the migration, activation state, and cellular interactions of Ag-specific T and B cells within lymphoid tissues. We have mainly focused upon the kinds of models we use within our lab and therefore predominantly discuss Ag-specific $CD4^+$ T cells, but similar studies have been carried out using $CD8^+$ T cells[74, 85, 106]. Secondly, we will discuss the development of novel fluorescence markers and imaging techniques which now also make it possible to follow the activation of signaling cascades, lymphocyte division, and cell-division associated phenotypic changes within an individual Ag-specific lymphocyte *in situ*.

PART 1: DETERMINING THE LOCATION AND NATURE OF THE CELLULAR INTERACTIONS UNDERLYING IMMUNE RESPONSES

T-B cell interactions

Generation of B cell responses and antibody production to most protein Ags requires Ag-specific T cells to become activated and help Ag-specific B cells. To become activated, T cells must be stimulated by Ag--laden antigen-presenting cells (APCs). Early studies suggested that dentritic cells (DCs) acquire Ag at the site of invasion or immunization and migrate to the parafollicular regions of lymphoid tissues, where they present Ag to naïve T cells[6, 23, 131]. More recently it has been shown that $CD11b^+$ DCs present Ag to naïve T cells which then produce interleukin (IL)-2, 6–18 h after Ag encounter[55]. However, the site of Ag acquisition by DCs remains unclear, although recent studies[57] have suggested that DCs acquire Ag both in the paracortex and at the site of immunization. After stimulation, T cells must then co-operate with Ag--stimulated B cells to promote B cell clonal expansion and antibody production. Although the compartmentalization and general regulation of germinal center formation was known[20, 70, 92], the location of Ag-specific T and B cell interactions was difficult to determine due to the extremely low frequency of the cells involved. However, using the adoptive transfer system the steps involved in this process have been greatly elucidated. It appears that after stimulation by APC in the paracortex, Ag-specific T cells proliferate in T cell areas, then move towards B cell follicles[36, 43, 69, 163]. This migration of lymphocytes is likely to be controlled by chemokines. It has been proposed that naïve T cells express the chemokine receptor CCR7, the ligands of which, secondary lymphoid tissue chemokine (SLC) and EBI-1 ligand chemokine (ELC), are produced by stromal cells in the T cell area of the lymph node (LN)[22]. Furthermore, some types of DC have been shown to express ELC, which may facilitate DC-T cell interactions[29, 73, 105, 120, 124, 127, 157, 158]. Upon activation, T cells upregulate the

CXCR5 chemokine receptor. The ligand for CXCR5, B lymphocyte chemoattractant (BLC), is produced by follicular stromal cells[22] and may therefore be responsible for recruiting Ag-stimulated T cells to B cell areas. The chemokine and chemokine receptor, CCR7/CXCR4, have been shown to be essential for B cell entry into LNs[111]. Activated B cells are stimulated to move to the boundary of the T and B cell area[24, 26, 60, 90], possibly by following an ELC or SLC gradient[79, 128]. T and B cells specific for the same Ag meet at the border between the T and B cell area[25, 35, 36], where Ag-specific T cells then interact with Ag-specific B cells and move further into the follicles to support maximal B cell clonal expansion, antibody production and germinal center formation[36]. A caveat to this is a recent study showing T and B cell motion *in vivo* to be random and therefore possibly not governed by chemokine gradients[98]. However, the use of optical highlighting[95] may permit "labeling": of cells in particular locations and their detection after subsequent migration.

We have examined specialized subsets of T cells and how they interact with B cells. T helper (Th) cells can be divided into two subsets: Th1 cells, which produce IL-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-β, and Th2 cells, which produce IL-4, -5, -6 and -13 [1, 101, 108, 115, 123]. It has long been suggested that Th2 cells provide help for antibody responses, whereas Th1 cells support cell-mediated immune responses[107]. However, immunolglobulin (Ig)G2a is an IFN--γ-dependent isotype, suggesting that Th1 cells can also support antibody production. Using the double adoptive transfer system described above we have shown that polarized Th1 and Th2 cells provide help for B cell clonal expansion and antibody production *in vivo*. Furthermore, we have shown that Th1 and Th2 cells do this in a similar manner using both CD40-CD154 and ICOS-B7RP-1 interactions[132, 135]. These studies contrast with others that suggest that Th1 cells do not upregulate CXCR5 and are therefore excluded from B cell follicles and unable to support the differentiation of naïve B cells into germinal center cells[117, 119]. However, more recent studies with human cells suggest that upon activation, CCR7 expression is decreased on both Th1 and Th2 cells and CXCR5 is upregulated (although transiently) on both cell types[18].

IN VIVO AG PRESENTATION

The adoptive transfer of Ag-specific Tg T and B cells as described above has proven to be very effective and sensitive in tracking early activation events and expansion of Ag-specific cells[125, 133]. However, in order to understand how and why Ag-specific T cells are driven to differentiate towards Th1 or Th2 effector cells, memory or anergic cells, regulatory T cells that can control inflammation or pathogenic T cells that induce tissue damage, it also will be important to study APCs.

APCs are amongst the first cells that interact with Ag. They acquire Ag via phagocytosis, pinocytosis, or receptor-mediated transport[150], degrade it by means of complex enzymatic systems in the cascades of lysosomal compartments[143], and finally present it in immunologically relevant form to Ag-specific $CD4^+$ or $CD8^+$ T cells[164]. Depending upon its form, the acquisition of Ag can also affect the maturation and phenotype of the APC[8] via effects on expression of co-stimulatory and adhesion molecules[2], production of certain sets of cytokines and chemokines and some mediators of immune response (reactive nitrogen and oxygen intermediates, histamine), or apoptosis[110]. All of this in its turn affects differentiation of Ag-specific T cells and determines the outcome of the immune response[94]. For these reasons, the visualization of APC early after immunization will be crucial for understanding the outcome of immune responses and designing vaccination strategies and therapies to control inflammation. In this respect it is very important to understand which subpopulation(s) of APCs determine the outcome of immune responses, when and where APC-T cell interactions occur, and what cytokines and co-stimulatory molecules are involved. Initially, APCs were studied *in vitro* after *in vivo* loading of Ag using proliferation assays[116], or cytokine production as a readout of Ag-presentation. However, the quantity of DCs that can be obtained from lymphoid organs is very low, and these studies necessitate complicated methods of DC isolation, which can affect the cells and influence the results. To overcome these difficulties, some studies have employed the growth factor flt3 ligand, which increases the quantity of DCs *in vivo*. This has aided the study of the role of DCs *in vivo,* which have now been shown to be key players in priming and tolerance[155]. A number of studies have also investigated the role of particular DC subsets in immune responses. For example, it has been shown that Peyer's patch $CD8\alpha^-$ DCs produce IL-10, while $CD8\alpha^+$ DCs produce IL-12[9, 58, 59, 93, 118]. Furthermore, $CD8\alpha^-$ DCs, including those from Peyer's patch, have been reported to induce differentiation of Th2 cells that produce IL-4, IL-10 and transforming growth factor (TGF)-β, while $CD8\alpha^+$ DCs induce IFN-γ-producing Th1 cells[9, 58, 59, 93, 118]. However, a role for $CD8\alpha^+$ DCs in tolerance induction has also been suggested[9, 42]. Indeed, $CD8\alpha^+$ DCs have a limited capacity to induce T cell proliferation but enhanced ability to induce Fas-mediated apoptosis[139].

It is also possible to visualize peptide:MHC complexes using monoclonal antibodies which specifically recognize this complex[57, 122]. Using this approach we detected peptide:MHC on CD8α⁻ DCs in PLN after feeding Ag in both tolerogenic and immunogenic forms[76]. The use of genetically modified animals and blocking antibodies has provided valuable information regarding the molecular basis of T cell-APC interactions (i.e. co-stimulatory molecules and chemokines)[155]. However, *ex vivo* functional studies of the type described above are not always very efficient for a number of reasons. First, endogenous factors can interfere with, suppress, or compete with the activation of the cells which are used as the readout. This may happen due to negative feedback[126] or cytokine reciprocal interactions[129]. Secondly, as noted above, in order to obtain enough cells which present Ag loaded *in vivo,* sophisticated and/or expensive separation procedures are used which can modify the phenotype and functional activity of APCs. Thus, it would be preferable to track APC directly *in vivo*. Transfer of APC labeled with fluorescent dyes [82] or immunization with Ag conjugated to fluorescent dyes[33] allows the APC and Ag to be tracked directly *in vivo.* Such studies have confirmed that Ag is acquired very early (within one hour) after immunization by APC and this is accompanied by the early accumulation of T cells in the same areas of lymphoid organs. It was shown that DCs are probably the major APCs which, being loaded with Ag *in vivo*, initially contact with Ag-specific T cells [55]. However, the study of fluorochrome-labeled Ag trafficking *in vivo* is affected by Ag-degradation and may be not representative of Ag presentation. In order to elucidate Ag-presentation, attempts to make antibodies which can track Ag in an immunologically relevant form (i.e. which recognize MHC with peptide bound and which therefore "see" what the T cell "sees") were undertaken. With the help of an antibody specific to HEL peptide in the context of MHC it was demonstrated that B cells display Ag in immunologically relevant form in the regional lymph node as early as 4 and 6 h s.c immunization[122], while DCs express the antigenic peptide later and for a longer period of time[122]. We showed, using the same antibody, that antigen is expressed on CD8α⁻ DCs in peripheral LNs as early as 6 h after feeding, but that this is not accompanied with the up-regulation of conventional co-stimulatory molecules such as CD80 and CD86, possibly suggesting a mechanism for the systemic effects of oral tolerance[76].

More recent studies[57] have revealed in detail for the first time the participants in the sequence of events that leads from injection of antigen in the skin to the induction of immune responses in the draining lymph node. Thus, *in vivo* analysis of Ag distribution, co-stimulatory molecules expression, and cytokine production by flow cytometry can provide valuable information more relevant to understanding the early events in the immune response. However, these approaches still suffer from the drawbacks associated with *ex vivo* manipulation of APC which can artificially activate these cells. Furthermore, the distribution of cell subpopulations may not represent the real situation within the lymphoid organ, as there may be differential effects on the purification and viability of particular subsets. Finally, the localization and interaction of cells cannot be visualized by flow cytometry. Thus, the study of cells directly within tissues gives direct information about cell numbers, phenotype, and their localization and interactions. Unfortunately, many imaging approaches have been unable to provide the quantification, which is such a useful feature of flow cytometry. As we will see below, laser scanning cytometry represents an exciting tool which combines the localization capacities of confocal microscopy with the opportunity to quantify the data by means of fluorescence or immunohistochemistry[67, 68]. It gives direct information about cell numbers, phenotype and their localization and interactions.

IN VIVO EXAMINATION OF TOLERANCE INDUCTION

Whilst the immune system must be able to respond to invading pathogens, it must remain tolerant to self Ags, as well as to dietary Ags and commensal flora. Central tolerance is induced by deletion of auto-reactive T cells in the thymus (reviewed in[136]); however, several mechanisms of peripheral tolerance exist to ensure destructive inflammation is not induced against harmless extra-thymic Ags. Until recently, the exact mechanisms of peripheral tolerance induction were unclear. The mechanisms by which peripheral tolerance was induced and the effect of tolerance on T and B cells were unknown. However, by making use of the adoptive transfer of Ag-specific B and T cells, it has now become possible to examine these events in more detail.

One important mechanism of peripheral tolerance induction that our lab has examined in detail is that associated with oral Ag administration. It has been known for many years that the feeding of soluble Ag induces a systemic hyporesponsiveness of both humoral and cell-mediated immunity[37]. By examining Ag-specific T cell populations following oral administration of Ag it has recently been demonstrated that this tolerance is not a lack of response, but rather that oral Ag induces the activation and clonal expansion of Ag-specific CD4⁺ T cells with similar kinetics as Ag fed in a regime which does not induce tolerance[32,

[133, 138]. Oral Ag induces upregulation of the very-early activation antigen CD69 on Ag-specific $CD4^+$ T cells within hours of feeding[133] and these cells appear to go through an initial response similar to priming[53]. However, recent studies have demonstrated that despite proliferating in response to fed Ag, Ag-specific T cells which are tolerized *in vivo* undergo less division than T cells primed by oral Ag[133].

Importantly, T cells tolerized by oral Ag fail to provide help for B cell antibody production. Again, the use of Ag-specific T and B cells has aided examination of the reasons for this observation. Unlike primed T cells, OVA-specific $CD4^+$ T cells tolerized *in vivo* by oral Ag fail to migrate into B cell follicles following feeding, and thus cannot provide help for B cell antibody production[134]. Upon re-challenge with Ag in adjuvant, tolerized T cells are now able to migrate into B cell follicles but still fail to provide B cell help, demonstrating a functional difference between tolerized and primed T cells[134]. The reasons for these differences in T cell migration in tolerance compared with priming remain unclear. However, analyzing the expression of chemokine receptors and the functional responses to different chemokines which are important in T cell migration into B cell follicles as discussed above, it is hoped that we can further examine the differences in migration of tolerant T cells.

Several mechanisms potentially mediate oral tolerance, including the induction of anergy (defined as a state of Ag-specific unresponsiveness) and the generation of Ag-specific regulatory T_{reg} cells depending on the dose of fed Ag[34]. The adoptive transfer of Ag-specific cells has further helped address these issues. Initial reports demonstrated that oral tolerance induces anergy of Ag-specific $CD4^+$ T cells, as these cells were unresponsive to Ag-restimulation *in vitro*[146]. However, recent reports have also suggested that oral Ag can induce a population of Ag-specific $CD4^+CD25^+$ T_{reg} *in vivo* capable of inhibiting the proliferation of naïve T cells specific for the same Ag *in vitro*[141, 162], further suggesting that different mechanisms of tolerance are induced by oral Ag. Although the ability of orally induced T_{reg} to modulate bystander responses has not been examined, the adoptive transfer of two Tg T cell populations of different Ag specificity into the same recipient allows further investigation of this phenomenon by examining the response of naïve T cells when presented in the context of an Ag to which tolerance is already established. The work of Pape et al.[114] has demonstrated that induction of tolerance in OVA-specific $CD4^+$ T cells induced by intravenous injection of OVA does not abrogate the expansion of naïve HA-specific $CD4^+$ T cells even when both Ags are immunized together. However, the authors did not examine the functional response of the bystander population, and it is possible that tolerant T cells may be capable of actively suppressing naïve T cell function. Our preliminary results have suggested that oral Ag is able to induce bystander tolerance, but that this suppression is not due to inhibition of the clonal expansion of the bystander population (Millington et al., unpublished data).

Whilst the exact molecules involved in oral tolerance remain unidentified, a recent report using Ag-specific T cells deficient in IL-10 production or CTLA-4 expression has suggested that whilst Ag-specific IL-10 production is not essential for tolerance, expression of CTLA-4 by Ag-specific $CD4^+$ T cells is required[32]. Thus, by crossing TcR Tg mice onto gene deficient animals, the role of individual molecules associated with suppression or migration can be assessed directly. Further assessment of molecules associated with tolerance and/or suppression is possible by analyzing Ag-specific cells purified (either magnetically or by FACSorting) at various stages following the induction of tolerance *in vivo*. Use of molecular techniques can then assess the expression of specific genes that may be associated with tolerance, or to examine for novel molecules associated with suppression using differential display (Eaton et al., unpublished data) or genome or proteome analysis[66].

Thus, tracking lymphocytes *in vivo* following tolerance induction has furthered our understanding of peripheral tolerance induction, demonstrating that tolerance is an active process, providing an insight into mechanisms that might be involved in tolerance, as well as allowing examination of the functional differences in how cells interact in tolerance and priming. The use of these technologies will remain an important part of studies into peripheral tolerance in the future.

PART 2: IMPROVEMENTS IN FLUORESCENCE IMAGING TECHNIQUES

The development of new fluorescence imaging techniques, such as multicolor video microscopy, laser scanning cytometry, and multiphoton microscopy, as well as standard fluorescence techniques, i.e. flow cytometry and confocal microscopy, have been vital in increasing our understanding of the cellular interactions which control immune responses. Advances in immunofluorescence techniques have led to the elucidation of the roles of signaling cascades and the ability to track *in vivo* lymphocyte division and apoptosis in the context of an Ag-specific immune response. Within the next sec-

tion we will discuss how recent developments in these techniques have provided new ways in which researchers can track the cellular changes occurring within Ag-specific lymphocytes *in vivo*.

SIGNALING CASCADES

Understanding intracellular signaling networks and how individual pathways transmit signals along linear tracts to result in the regulation of discrete cell functions will be fundamentally important for the design of new vaccines and therapies for disease[49, 87]. The cellular and molecular mechanisms that control immune responses are principally due to the changes in intracellular signaling cascades, whether it leads to cell cycle arrest, a change in cellular phenotype, or an increase in the expression of a pro-inflammatory cytokine. Historically, the activation of signaling molecules had been elucidated using transformed cell lines and Western blotting analysis, and the majority of our knowledge of signaling pathways to date results from such approaches[54]. Studying signaling cascades in the physiological microenvironment where cellular interactions occur *in vivo* is likely to be the most accurate method to dissect signaling differences that underpin disease and hence provide new insights necessary for groundbreaking advances in therapies.

More recently, disrupting signaling cascades in Tg mice has illustrated ways in which defects in signaling pathways in both T[109] and B cells[80] can contribute to particular types of immune responses. For example, the development of autoimmune diseases is a complex process with cellular outcomes such as cytokine production, cell-cycle progression, and cellular differentiation all linking to intracellular signaling cascades. One molecule which may be centrally involved is phosphoinositide 3-kinase (PI3K), which is a key molecule involved in phospholipid metabolism and regulates many cellular events such as cell growth and survival[153]. Genetically modified mice have linked the PI3K pathway to autoimmune susceptibility, possibly by both promoting T cell survival and regulating T cell proliferation.

Recent advances in immunofluorescence imaging techniques have also led to a single cell approach in revealing the early activation of PI3K at the immunological synapse between T cell and APC, almost immediately after first contact[45]. The immunological synapse concentrates both TcR and antigenic MHC--peptide complex in the center of the APC-T cell contact. Using a combination of fluorescent-specific probes, confocal microscopy, green fluorescent protein (GFP) fusions, and T cell hybridomas, it is now possible to study a plethora of signaling cascades, costimulatory molecules, and structural components in the formation of the immunological synapse after Ag recognition[5, 14, 154].

Following TcR engagement, coordinated assembly leads to formation of an immunological synapse or supramolecular activation cluster (SMAC). The central zone, c-SMAC, contains the TcR and surface accessory molecules such as CD4, CD2 and CD28. Surrounding the central zone is a second peripheral adhesion ring, p-SMAC, which is enriched for the integrin, LFA-1, and the cytosketal molecule talin[40]. Phospho-specific antibodies have been used to recognize the activated forms of the key TcR regulated cytoplasmic tyrosine kinases such as Lck and ZAP-70, therefore demonstrating the timing and the location of these early TcR activation events within the immunological synapse[83].

Preparing gene fusions of the various molecules with GFP and observing their movements during T cell recognition using multicolor video microscopy[77] have eloquently shown the dynamic nature of ligands involved in the immunological synapse. For example, initially CD4 and TcR-associated CD3ζ cluster within the center of synapse and then CD4 moves to the p-SMAC[78]. The significance of the redistribution of CD4 is not known.

Although such *in vitro* approaches have played a vital role in defining potential signaling pathways and dynamic cell changes, it could be speculated that the microenvironment where cell activation occurs *in vivo* may be different from that within *in vitro* systems. With the ability to track Ag-specific T cells *in vivo*, it is now possible to study signal transduction under physiological conditions[159, 160]. Flow cytometry has been used to monitor Ag-induced c-jun and p38 mitogen-activated protein kinase phosphorylation in individual $CD4^+$ T cells. Results contradicted previous *in vitro* work that Ag-induced c-jun and p38 mitogen-activated protein kinase phosphorylation are dependent on CD28 signals[88, 161], hence highlighting the importance of verifying signaling pathways *in vivo*. At present, we are establishing methods to directly quantify the activation of signaling molecules[7, 28] within Ag--specific T cells in secondary lymphoid tissue using the Laser Scanning Cytometer (LSC).

Already it is possible to monitor IL-2 production within Ag-specific T cells *in vivo* using a gene targeting "knock-in" approach, integrating GFP into the endogenous IL-2 locus[104]. Therefore, the next logical step would be to use this strategy to track the cellular location and activation of signaling molecules within

an Ag-specific immune response *in vivo*. Theoretically, with new techniques such as multiphoton microscopy[15, 99, 137] it should be possible to detect these genetically manipulated Ag-specific T cells and/or B cells whilst they interact with different cell types within a lymphoid tissue in real time *in vivo*. These approaches will markedly expand our understanding of the immune system in both time and space and put the detailed body of *in vitro* molecular and cellular studies into a more physiological context.

LASER SCANNING CYTOMETRY

Current flow cytometric technology allows quantitative assessment of surface and intracellularly expressed molecules on isolated cells. However, the need to disrupt tissues prevents correlation of this expression with anatomical location. In contrast, immunohistochemistry in conjunction with conventional or confocal microscopy allows localization of staining, but little in the way of quantification. The recently described LSC [67,68] allows a combination of both approaches, as it can apply flow cytometric laser technology to intact tissue. The LSC automatically measures laser-excited fluorescence at multiple wavelengths and forward light scatter from slide-based individual cells or tissue sections which have been treated with one or more fluorescent dyes in order to rapidly determine multiple cellular constituents. The fluorescence measured by the LSC can be further localized within the cell to the nucleus or the cytoplasm. The LSC also records the position of every cell on the slide so that interesting populations of cells can be relocated and re-visualized.

Using LSC we have begun to determine the level of expression of a variety of T cell-expressed surface and signaling molecules and are relating this to the anatomical location of T cells in different compartments within lymphoid organs. Such approaches will allow us to relate differences in the expression of such molecules to the function of T cells in different sites. For example, it is known that ICOS-B7RP-1 interactions have an important role in T cells helping B cells[21, 130], but it remains unclear where this interaction occurs within the lymph node. Controversy also exists regarding the location of T cells providing B cell help. Smith et al.[135] showed that Th1 and Th2 $CD4^+$ T cells provide help by migrating into B cell follicles. In contrast, others have suggested that directly assessing cytokine expression levels of Ag-specific $CD4^+$ T cells within the follicle will be required to confirm the differentiation state of these cells in this location. Thus, assessing Th1/Th2 cytokine expression levels of Ag-specific $CD4^+$ T cells at several time-points in different locations in the lymph node could aid in discovering when and where in the lymph node T cells differentiate into Th1 and Th2 T cells and where they then help B cells. Doing this would establish if T cells differentiate into Th1/Th2 cells before they home to the follicle, where they then provide B cell help at the edge of the follicle, or whether some interaction in the follicle itself leads to differentiation of the T cells into Th1/Th2 cells before they provide B cell help further inside the follicle. If the location of T cell differentiation into Th1/Th2 cells were identified, then this might indicate which cell types could be involved, i.e. outside follicle:DCs or activated T cells, inside follicle:activated T cells or B cells, and therefore may suggest some chemokines that are possibly involved. As noted above, it has been shown *in vitro* that stimulated B cells up-regulate their responsiveness to ELC and SLC (ligands for T cell chemokine receptor CCR7) as well as their expression of several T cell chemoattractants and that stimulated T cells increase their expression of CXCR5 and up-regulate their responsiveness to the ligand for CXCR5, indicating that both cell types alter themselves after stimulation in such a way as to encounter each other at the edge of the B cell follicle[22]. This upregulation of CXCR5 has also been shown on Ag-specific $CD4^+$ T cells *in vivo* under immunization conditions that drive the migration of T cells to the follicle[4].

However, much of this evidence remains circumstantial. A direct assessment of chemokine receptor expression on Ag-specific T and B cells during a detailed time course in a LN *in vivo* will be the only way to definitively determine the role of these (and other) molecules. This could be achieved with a combination of the adoptive transfer and LSC approaches described above. Tissue maps depicting the locations of B cell follicles and Tg T cells within the LN can be generated and the number of Ag-specific T cells in the follicular areas and paracortex can be analyzed. The levels of expression of ICOS, Th1/Th2 cytokines, chemokine receptors, etc. on Ag-specific T cells in different locations can be quantified. Regions can be drawn around follicles of interest and the LSC can relocate to them, enabling image analysis of these areas. The captured images can then be linked to the corresponding area on the tissue map allowing correlation of quantitative and qualitative data with anatomical localization. This ability of the LSC indicates its potential as a tool for analyzing immune responses at the cellular and molecular level *in vivo*.

MEASURING LYMPHOCYTE DIVISION *IN VIVO*

CFSE labeling of Tg lymphocytes

Fluorescent dyes such as 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) have been wide-

ly exploited in recent years to study diverse aspects of *in vitro* and *in vivo* cell growth and function[38, 46, 91]. CFSE permeates cellular membranes and attaches to the free amine groups of cytoplasmic proteins whereupon it segregates equally between daughter cells at mitosis. This results in the sequential halving of fluorescence intensity in progeny cells when measured by flow cytometry[91]. Cell activation, differentiation and migration appear to be unaffected by CFSE incorporation and hence this labeling technique has greatly facilitated the study of lymphocyte biology, migration and fate *in vivo*, particularly when used in conjunction with the adoptive transfer of receptor Tg T and B cells. Fluorescently labeled naïve lymphocytes from donors expressing Tg TcR or BcRs migrate to lymphoid organs following transfer and upon Ag-driven activation undergo a series of phenotypic changes and acquire functions that are cell division dependent. CFSE-labeled Tg lymphocytes identified using clonotypic Abs or Abs-specific for donor-derived surface markers (e.g. TcR chain, alternative Thy-1 alleles, surface IgM^a) can be further studied for expression of cell surface molecules (including activation markers CD69, CD25, markers of memory phenotype), intracellular cytokines, or surface-bound antibody. Lymphocyte cell surface marker expression, T cell cytokine production, B cell isotype switching, and lymphocyte apoptosis are all highly division-dependent processes[10, 27, 38, 46, 47] and these techniques when used with CFSE labeling have allowed identification of many key factors that influence priming of the adaptive immune response. Applications of this technology include (a) studying factors that influence Tg lymphocyte division and clonal expansion *in vivo*, (b) studying activation and the acquisition of effector and memory cell characteristics, (c) identifying the cells responsible for presenting Ag to naïve T cells, (d) identifying the location and duration of Ag presentation, and (e) tracking lymphocyte migration into and out of a particular site *in vivo.* Some specific examples are shown below.

Factors influencing lymphocyte clonal expansion

Upon stimulation, lymphocytes progress through several rounds of division in a process referred to as clonal expansion. During this process naïve lymphocytes differentiate into specialized effector and memory cells[64]. However, many of the factors that control the progression and duration of clonal expansion are not clear. Because the magnitude of the initial clonal burst influences the size of the effector and memory cell pools, identifying factors that influence clonal proliferation are important for the design of effective therapies and vaccines[65]. CFSE-labeling of Tg $CD4^+$, $CD8^+$ T cells and B cells prior to adoptive transfer has identified some of the numerous factors that influence this process. Ag dose, form and the presence of "danger signals" have all been shown to influence *in vivo* cell proliferation or clonal expansion of Tg lymphocytes. Recent studies[84] using CFSE-labeled DO11.10 $CD4^+$ T cells have shown that Ag dose influences the number of $CD4^+$ T cells activated but not the rate of cell proliferation, and that even after brief Ag exposure, $CD4^+$ T cells are programmed to divide independently of any further antigenic stimulus. Similarly, Bonnevier and Mueller[13] demonstrated that the Ag dose regulates the rate of entry into cell cycle but not the overall number of divisions per cell. Similar studies have been performed using CD8 T cells[63, 148]. Furthermore, CFSE-labeling of DO11.10 $CD4^+$ T cells prior to adoptive transfer has allowed us to show that the lower level of clonal expansion of $CD4^+$ T cells associated with feeding Ag in a tolerogenic rather than an immunogenic form is associated with a defect in T cell division and not a deficit in cell activation[133].

Clonal expansion of Tg lymphocytes following adoptive transfer and Ag-driven activation is measured as an increase in the percentage of the Tg population relative to the total lymphocyte pool. In some cases, however, particularly where Ag may be limiting (e.g. early stages of viral infection, DNA vaccination), it is not possible to demonstrate significant expansion of Ag-specific T or B cells[125]. In these cases cell division tracking has proven to be a particularly useful and sensitive strategy. Our own studies have employed CFSE labeling techniques to study $CD4^+$ and $CD8^+$ T cell priming following DNA vaccination and factors that influence these priming events[125]. In this case we found that relatively simple construct manipulations have a profound effect on the ability of DNA vaccines to activate and induce the division of $CD4^+$ and $CD8^+$ T cells.

CFSE LABELING TO STUDY LYMPHOCYTE DIFFERENTIATION

Cell division and effector/memory phenotype

Because cell division is an important parameter that determines the differentiation of T and B cells, several groups have attempted to correlate division number with the acquisition of specific effector and memory cell characteristics, including cytokine production, antibody production, and cytotoxic activity. *In vitro* experiments[10, 27, 38, 46, 47] have shown that T cells acquire the ability to express IFN-γ, IL-4 and other cytokines in a division-dependent manner. Analysis of the cytotoxicity of Tg $CD8^+$ T cells from transferred, immunized mice has shown that as with

cytokine production, sorted cells that have undergone more divisions *in vivo* were more cytotoxic in conventional *in vitro* cytotoxic T lymphocyte (CTL) assays[31]. As discussed below, the modulation of surface receptor expression determines the ability of effector cells to enter non-lymphoid tissues and this can also be studied by CFSE labeling. The migratory behavior of lymphocytes is determined by alteration in cell surface molecule expression. In most cases Ag--driven activation and cell division are accompanied by up- or down-regulation of surface markers such as CD69, CD25, CD62L, many of which influence migration within the lymphoid tissue and entry into non-lymphoid sites.

Ag presentation in vivo: site, Ag persistence, and APC

CFSE labeling of T cells can be useful for identifying both the anatomical site and duration of Ag persistence following immunization[100] or in Tg models of autoimmunity (e.g. RIP-mOVA mice[81]. Various lymphoid organs can be examined for the presence of dividing cells, which are indicative of local Ag presentation. Furthermore, analysis of cell division in lymphocytes transferred at different times post-immunization can be used to assess Ag or peptide/MHC persistence[100]. The cell type responsible for presenting Ag to naïve T cells can also be studied using labeled-cells. Another approach is to employ bone marrow chimeras[11], where Ag expression is restricted to either bone marrow-derived or parenchymal cells. B cells can also be labeled prior to adoptive transfer. In one example, CFSE-labeled Ag--loaded Tg B cells have been used to show that in addition to direct presentation to naïve T cells, Ag--specific B cells efficiently transfer Ag to CD8α^+ DCs *in vivo* for presentation to T cells[144].

LOCATION OF T CELL PROLIFERATION AND LYMPHOCYTE MIGRATION PATTERNS

Upon Ag-induced activation in lymphoid organs, T lymphocytes acquire, through a division-related differentiation process, surface selectins, integrins and chemokine receptors that are not expressed on naïve T cells. These surface molecules determine the migratory behavior of effector cells out of lymphoid organs and into nonlymphoid tissues such as skin, lung, liver and intestine. Once in nonlymphoid tissues CD4$^+$ effector T cells produce cytokines that may augment the antimicrobial function of phagocytes or, in the case of CTLs, may directly kill infected cells. Recent studies have shown that Ag-specific CD4$^+$ T cells proliferate in LNs draining the Ag injection site, and cells that have undergone the most divisions acquire the ability to bind CD62P, a molecule present on inflamed blood vessels[121]. Expression of this CD62P ligand (PSGL-1) allows these cytokine-producing lymphocytes to migrate to and preferentially accumulate at the injection site. Hence homing molecule expression is closely linked to cell division.

The dissemination of the immune response from LNs draining the infection or injection site to secondary lymphoid tissues can also be studied using CFSE--labeled T cells. For example, HSV-1 gB-specific CD8$^+$ T cells were labeled prior to adoptive transfer, and following HSV-1 infection, gB-specific cells in the local LN had divided and were cytolytic as early as day 2 post-infection[19]. In contrast, proliferating T cells were not detected in the spleen until day 4, at which time they had undergone extensive cell division, suggesting that proliferation was occurring in the secondary lymphoid compartments in an Ag-dependent fashion.

CFSE-labeling for imaging lymphocyte behavior in tissues and intact LNs

Because CFSE displays similar emission and absorption characteristics to fluorescein, labeled cells can be visualized and tracked using conventional fluorescence microscopy. In addition, labeled T cells within intact LNs have been visualized before and after Ag stimulation using multiphoton laser microscopy[98, 99]. Experiments such as these provide valuable insight into the dynamic behavior of lymphocytes *in vivo*, as *in vitro* systems cannot replicate tissue environmental influences.

ANALYSIS OF APOPTOSIS

Apoptosis plays a crucial role in maintaining homeostasis within the immune system. During lymphocyte development, self reactive T and B cells are removed by an apoptotic mechanism, thus preventing potential autoimmune diseases such as systemic lupus erythematosus (SLE)[140], where antibodies to self Ags result in huge amounts of immune complex formation. SLE has been linked with increased levels of soluble Fas that inhibits proper apoptosis of the self reactive lymphocytes[102]. Apoptosis also functions subsequent to the expansion phase of the lymphocyte response when the contraction phase is initiated in the expanded population. This contraction is mediated by apoptosis and ensures the prevention of diseases such as autoimmune lymphoproliferative syndrome (ALPS), which is characterized by defective lymphocyte death[12] resulting in lymphadenopathy and increased occurrence of lymphoma due to lack of cell death. ALPS is also associated with an increase in autoimmune disorders resulting from failure to clear self reactive lymphocytes.

Apoptosis allows removal of lymphocytes in a non--inflammatory manner, which also helps to prevent autoimmune reactions. It does this by preventing the release of self Ags because engulfment occurs prior to lysis of the apoptotic cell, which also prevents the formation of a pro-inflammatory environment in which encountered Ags would be responded to as though foreign and an immune response mounted rather than tolerance being initiated. Furthermore, macrophages taking part in the engulfment process actively secrete anti-inflammatory mediators such as IL-10 and TGF-β[30].

Apoptosis is employed within the context of development of the immune system, maintenance of the immune system, and action of the immune system. The development and maintenance of the immune system and apoptosis have been outlined above; however, regarding the presence of apoptosis in the action of the immune response, we mean within the germinal center reaction and as a killing mechanism. Developing B cells form germinal centers where maturation of the population occurs to produce a more effective and specific population of B cells. The surplus B cells are removed by apoptosis[44]. Also in the action of the immune system, apoptosis is observed as it acts in the context of cytotoxic lymphocyte killing of the target cell. Cytotoxic T cells do this by initiating the caspase cascade within the target cell by releasing granzyme B into the target cell. Granzyme B mimics the activity of the pro-caspase proteases[142] and thereby initiates the apoptotic process.

There are some universal features of apoptosis observed in cells undergoing apoptosis. These include the classical defining morphological changes[71] (including membrane disruption and formation of apoptotic bodies) in addition to chromatin condensation and cleavage[156], mitochondrial membrane potential disruption[75], and caspase activation[86]. These changes are the result of the activation of a family of cysteine-dependent aspartine-specific proteases that are called “caspases”. These activated proteases proceed to cleave host cell structural and regulatory proteins along with nuclear DNA[61]. Caspase activation leading to apoptosis is initiated by two different pathways[50]. The first signals through various surface receptors, most notably Fas/Fas ligand and the TNF receptor, via an adaptor protein such as FADD and TRADD, which contain “death domains”. These adaptor molecules then recruit the caspases. This route is functional in the control of self reactive lymphocytes. The second route to caspase activation involves the release of “Bim” from microtubules by an unknown apoptosis-inducing stimulus that causes mitochondrial breakdown and activation of caspases. Most significantly this mechanism functions in the contraction phase of the immune response.

Current detection methods of apoptosis rely upon these universal features. Visualization of morphological changes is still used in some instances to detect apoptosis; however, the membrane disruption[96] associated with these morphological changes is often detected using Annexin V (conjugated to a fluorescent marker), sometimes in association with a vital dye such as propidium iodide. Annexin V binds to phosphatidylserine which is exposed on the cell surface during early apoptosis, thereby allowing detection of early apoptotic cells using fluorescent microscopy or flow cytometry[145, 149].

The chromatin condensation and cleavage described above can be detected using a variety of techniques. By staining DNA within a cell using a fluorescent stain and then observing the fragments that are subdiploid using flow cytometry or fluorescent microscopy, apoptosis can be confirmed. Running cells lysates upon an agarose gel also allows the detection of cleaved DNA, although this is not a quantitative indicator. More common is the use of TUNEL (terminal deoxynucleotidyl-transferase dUTP nick end labeling) kits which use fluorescently labeled Br-dUTP to label exposed 3’-hydroxyl ends formed as a result of DNA cleavage. Detection of the degree of fluorescence can be quantitative using a flow cytometer[89, 151].

Measurement of mitochondrial membrane disruption by dyes that produce a color shift dependent on membrane potential allows the detection of the mitochondrial involvement in apoptosis along with providing a standard determinant of the presence or absence of apoptosis.

Finally, caspase activation and involvement can be measured by a number of methods[17]. Caspase inhibitors are commonly used to determine the dependency of the apoptosis being observed upon caspases. Inhibitors specific for a certain caspase or of a more general nature are available. Similarly, antibodies specific for specified caspases allow the activation status and presence of these caspases to be identified.

The expression of a number of cell surface proteins is also frequently used as an indicator of apoptosis[112]. Most prominently, the expression of Fas and Fas ligand upon activated T cells and natural killer cells can be measured by a variety of methods, including Western blot and flow cytometry.

Many of the above techniques can be used in conjunction with flow cytometric or laser scanning cytometric identification of Ag-specific lymphocytes to allow the measurement of the degree of apoptosis within the population of Ag-specific lymphocytes. Detection of location and clonal expansion of Ag specific lymphocytes has aided the study of various aspects of the lymphocyte biology, so the ability to measure the death of lymphocytes can be used in a similarly informative manner to elucidate various aspects of lymphocyte biology. The examples below show how tracking the different roles of apoptosis of Ag specific lymphocytes has informed us of some important things.

As outlined in the introduction to this section, apoptosis plays a key role in the selection of lymphocytes during their development to eliminate self reactive or defective cells. Wang and Yang-Xin[152] recently used a TcR Tg system to elucidate the role of the ligand LIGHT in thymocyte negative selection. By making LIGHT Tg mice and crossing them to H-Y TcR Tg mice they were able to demonstrate that in the presence of H-Y Ag, LIGHT Tg mice have increased levels of thymocyte apoptosis as measured by annexin V/PI compared with normal H-Y Tg mice. They were thus able to show that LIGHT was TcR linked and was directly involved in thymocyte selection.

In 1998 Van Parijs et al.[147] demonstrated the different role of receptor-mediated apoptosis and mitochondria-mediated apoptosis in the induction of apoptosis in T cells depending on the source of Ag. They utilized a TcR Tg specific for HEL Ag and by expressing HEL as a self Ag were able to demonstrate that Fas--mediated death is important for peripheral deletion. Similarly by immunizing with HEL they were able to demonstrate that Bcl-2 is important for the contraction phase in this response. Although they never measured apoptosis *in vivo*, measuring the level of apoptosis *in vitro* was an important part of their demonstration that the T cells they were using were normal.

More recently Grayson et al.[41] used identification of apoptosis in Ag-specific $CD8^+$ T cells to elucidate the mechanism of apoptosis during the contraction phase after a physiological response to LCMV. By measuring the mitochondrial potential in Ag specific cells after viral infection they were able to demonstrate that decreasing mitochondrion potential and, therefore, increased membrane permeability was responsible for the apoptosis observed during the contraction phase. In addition they were able to demonstrate that caspase activity begins earlier than previously expected, i.e. when infection is still present, and also that memory cells have a low mitochondrial membrane potential and yet are not apoptotic.

As a killing mechanism, apoptosis induction by Ag--specific lymphocytes was observed to occur by Ando et al.[3]. By expressing hepatitis B surface Ag as a self Ag, TcR Tg mice for this Ag were observed to induce apoptosis of the target cell.

An example of this process going wrong and autoimmunity being induced is diabetes. The factors influencing the development of diabetes were investigated by Hill et al. [51]. By adoptively transferring islet specific $CD4^+$ T cells and measuring apoptosis of this population it was demonstrated that the acceleration or deceleration in speed of onset of diabetes was not due to the proliferation and apoptosis of these islet specific $CD4^+$ T cells, but rather due to the rate of entry of specific cells into the pancreas.

Finally, determining the mechanism of action of therapeutic agents may also involve measuring apoptosis. Holcombe et al.[52] determined the effect of the immunosuppressive agent 15-deoxyspergualin *in vivo* by measuring its effect upon $CD4^+$ T cells specific for MBP. By demonstrating that DSG inhibits cell proliferation, they then used the annexin V/PI assay to demonstrate that DSG does not accomplish this by inducing apoptosis.

Thus, this manuscript illustrates that with recent advances in immunology and imaging we will be able to employ identifiable, antigen-specific lymphocytes and APCs with reporter gene identifiable functions in conjunction with techniques such as multiphoton microscopy and optical highlighting to definitively determine where, when and how these cells interact in the physiological context *in vivo* in tolerance, priming, differentiation of Th1 versus Th2 cells, disease induction and in many other situations.

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