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Secondary Synaptic T-T Circuits Drive Protective CD8⁺ T cell Differentiation

Audrey Gérard¹, Omar Khan¹, Peter Beemiller¹, Erin Oswald¹, Joyce Hu², Mehrdad Matloubian², and Matthew F. Krummel¹

¹Department of Pathology, University of California, San Francisco, 513 Parnassus Ave, HSW512, San Francisco, CA 94143-0511, USA

²Division of Rheumatology, Department of Medicine, University of California, San Francisco, 513 Parnassus Ave, HSW512, San Francisco, CA 94143-0511, USA

Abstract

Immunization results in the differentiation of CD8⁺ T cells, such that they acquire effector capabilities and convert into a memory pool capable of rapid response upon re-exposure. The initial priming of T cells takes place via an immunological synapse (IS) formed with an antigen-presenting cell (APC). By disrupting synaptic stability at different times, we show that CD8⁺ T cell differentiation requires cell interactions beyond those made with APC. We identify a 'Critical Differentiation Period' (CDP) characterized by and requiring the interaction between primed T cells. We show that T-T synaptic interactions play a major role in the generation of protective CD8⁺ T cell memory. T-T synapses allow T cells to polarize critical interferon- γ secretion towards one another. "Collective" activation and homotypic clustering therefore drive private cytokine sharing and act as regulatory stimuli for T cell differentiation.

Introduction

Effective adaptive immunity relies on the capacity of lymphocytes to differentiate and to make a concerted response. An immune response requires a few specific T cells not only to find rare cognate antigen (Ag)-presenting cells (APCs), but also to receive appropriate signals to differentiate into effector or memory subsets. Much work has focused on figuring out how the appropriate level of antigen, its affinity for the TCR, or the requirement of costimulation during a priming APC encounter regulates optimal T cell differentiation. However, proper CD8⁺ T cell differentiation requires other signals, like CD4⁺ T cell help, and cytokines^{1–3}. Despite considerable work, the timing, site and conditions of CD8 differentiation remain unknown^{3–5}.

Correspondence should be addressed to AG (Audrey.gerard@ucsf.edu) or MFK (Matthew.krummel@ucsf.edu)..

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Priming of CD8⁺ T cells occurs in multiple ways, and the requirement for particular cytokines or costimulators may be overcome by alternate pathways⁴. As a result, populations of Ag-specific CD8⁺ T cells formed are heterogeneous⁶, and not all T cells, even ones bearing the same TCR, will evolve similarly. Despite some heterogeneity, CD8⁺ T cells mostly respond in an integrated manner, but how they coordinate their response is elusive. Furthermore, only a few T cells are required to mount an efficient and coordinated immune response, and high precursor frequency is not beneficial. Various lines of evidence suggest that T cells have developed strategies to find other activated T cells^{7,8}, to exchange information⁹ and to cooperate¹⁰.

Recent advances in 2-photon imaging have permitted direct observation of T cell behaviour during an immune response in lymph nodes (LNs). Following recognition of their cognate Ag presented by a dendritic cell (DC), T cells slow down and form long stable interactions with DCs^{11–14}. During this “arrest phase”, also called “Phase II”¹¹, several T cells are often found interacting with the same APC, forming clusters¹⁵. During clustering events, it has been noted that T cells might interact with each other^{16, 17}. *In vitro*, we have demonstrated that CD4⁺ T cells form synapses where increased localized interleukin 2 (IL-2) signalling complexes are found^{9, 18}. The implication of those interactions for T cell responses *in vivo*, however, has not yet been assessed.

Here, we provide evidence of a critical differentiation period (CDP) for CD8⁺ T cells during the course of an immune response. We demonstrate that cell-cell interactions beyond T-APC interactions are necessary, at physiological precursor frequencies, to generate optimal CD8 responses. We show that mutual adherence and synapses between T cells is one of the cell-cell interactions required for CD8⁺ T cell differentiation. T-T synaptic structures thus provide increased sensitivity to cytokine and are required for T cells to collectively interact.

Results

A Critical Differentiation Period for CD8 Differentiation

The time-course of CD8⁺ T cell activation in lymph nodes, in response to vaccination or infection, is characterized by distinct phases of cell motility (Supplementary Fig. 1a) and cell-cell interaction (Fig. 1a)^{11,12,19}. To follow CD8⁺ T cell behaviour relative to APCs during an immune response, RFP-expressing antigen-specific OT-I TCR transgenic T cells were adoptively transferred into hosts in which Cd11c was marked by YFP. Two hours after an immunization that targets dendritic cells (DC) as APCs, OT-I T cells intermingled in the antigen-presenting dendritic cell network but did not extensively dwell on any single DC (Supplementary Movie 1). By 10 hours, they had decelerated within an environment containing DCs (Supplementary Fig. 1a and Supplementary Movie 2). By 24 hours, OT-I cells were still moving at a low speed (Supplementary Fig. 1a), but in addition, they were now often in clusters with other activating T cells (Supplementary Movie 3). Clustering at some phases but not others provides further subdivision of the characteristics of motility arrest¹¹. By 60 hours, T cells resumed their migration (Supplementary Fig. 1a and Supplementary Movie 4). T cell-APC synapses formed in the first hours of DC encounter are known to be sufficient for TCRs to cluster and internalize²⁰ and for T cells to up-regulate the TCR-driven activation marker, CD69 (Supplementary Fig. 1b and ^{11, 19, 20}).

To address the requirement for adhesive synaptic contacts throughout the time course of the response, we injected LFA-1 antibodies (LFA-1 Ab) into mice to block these interactions. Blockade was initiated at times corresponding to the transient (2 h), clustered (24 h) and very late time points (60 h)^{11,12,19} (Fig. 1b). LFA-1 Ab administered 2 hours after antigen-administration reduced proximal CD69 up-regulation at 32 hours by ~30% and the extent of T cell differentiation exemplified by interferon- (IFN-) production at day 6 by ~60% (Fig. 1b, Supplementary Fig. 1b,c). This result is consistent with adhesive synapses broadly facilitating both priming and differentiation. In contrast, LFA-1 blockade started at 24 hours had no effect on expression of the activation marker CD69 at any subsequent time (Fig. 1b, Supplementary Fig. 1b) or proliferation (Supplementary Fig. 1d). However, this 24 h blockade resulted in a ~60% decrease in the induction of IFN- production by OT-I cells compared to controls by day 6, suggestive of a different requirement for differentiation as compared to priming. Due to experimental constraints, we adopted 1×10^6 OT-I T cells into wild-type recipients for early assessment of CD69 up-regulation at 32 hours as this is required to recover sufficient cells, but we adopted just 5000 OT-I T cells to measure IFN- expression as these numbers are more physiologically accurate^{21,22}. We confirmed that differentiation still required LFA-1-dependent interactions 24 hours post-immunization using higher precursor frequency, although non-physiological precursor frequency alone increases differentiation regardless of blockade (Supplementary Fig. 1e). Blocking LFA-1-dependent interactions 60 hours post-immunization had no effect on IFN- production (Fig. 1b and Supplementary Fig. 1c), suggesting there is a short time window (24–60 h) when T cells integrate differentiation cues in a cell contact-dependent manner.

We observed similar evidence for an integrin requirement using a different adjuvant-immunization model with a delayed blocking of ICAM-1, the ligand for LFA-1 (Supplementary Fig. 2). This indicated that inhibition of differentiation was not due to LFA-1 Ab-modulated signalling nor block of T cell trafficking. Finally, because LFA-1 Ab treatment could function by blocking late entry of new OT-I cells in LNs and thus potentially affect memory through different cells being activated, we synchronized T cell homing to LNs by treating the mice with CD62L Ab to block new entry²³. CD62L Ab treatment did not affect IFN- production by OT-I cells, and LFA-1 Ab treatment in the context of CD62L blockade still resulted in inhibition of IFN- production (Supplementary Fig. 1f). This result confirms that inhibition of CD8 T cell differentiation by LFA-1 Ab treatment was not due to inhibition of T cell homing or inhibition of late T-APC encounter.

We also observed similar reductions in both the yield and number of antigen-specific CD8⁺ T cell effectors and the proportion of antigen-specific cells that were IFN- positive in response to sub-lethal bacterial (Fig. 1c,d and Supplementary Fig. 3c) or viral (Fig. 1e,f and Supplementary Fig. 3d) infections. For those experiments, we adjusted the timing of LFA-1 Ab treatment in accord with the time when markers of TCR-driven activation were maximal: 32 and 16 hours, respectively (Supplementary Fig. 3a,b). Finally, we observed a similar requirement for secondary adhesive contacts when a non-TCR transgenic responder mouse (Yeti mouse) was assayed, utilizing peptide-MHC pentamers to identify CD8⁺ T cell responders in the endogenous repertoire and using YFP as a read-out of IFN- transcription (Fig. 1g,h and Supplementary Fig. 3e). These data provide evidence for a late requirement

for integrin-mediated interactions, during a variety of immunological challenges, and at physiological precursor frequencies.

CDP Interactions Mediate Protective Memory

As these data indicated a distinct critical differentiation period (“CDP”) for integrin-mediated engagements, we also tested the consequence of “CDP-blockade” (LFA-1 Ab treatment after TCR-pMHC dependent CD69 up-regulation was established) for memory establishment and successful vaccination. During LCMV infection of WT hosts adoptively transferred with a low number of P14 TCR transgenic T cells, CDP blockade resulted in a decrease of P14 cell numbers 2 weeks after challenge (Fig. 2a). Furthermore, the balance between SLECs (short-lived effector cells) and MPECs (memory precursor effector cells) was altered by CDP blockade (Fig. 2b). Blocking LFA-1-dependent interactions during the CDP led to a decrease in the percentage of SLECs and conversely, an increase of MPECs at dpi 15. However, of those MPECs, the percentage of cells displaying a central memory phenotype was greatly diminished, suggesting that establishment of long-lasting memory was impaired (Fig. 2c). This observation held true for CDP blockade after DEC-OVA immunization, which resulted in a diminution of the percentage of OT-I cells with central memory phenotype as assessed by CD62L and CD44 positivity at day 8 (28.37% for control mice and 12.28% for LFA-1 treated mice, Fig. 2d). As a consequence of blockade, we also recorded a reduction of IFN- γ producing cells among the cells recovered 3 days after recall experiments (Fig. 2e), as well as a reduction in the percentage of recovered OT-I cells (Fig. 2f). These data suggest that successful vaccination would rely on efficient CDP. We modified an established DC vaccination protocol against *Listeria*-expressing ovalbumin²⁴, and blocked LFA-1-dependent interactions during the CDP to establish the relevance of this period for protection. Normal vaccination conferred protection against a lethal dose of LM-OVA to 80% of mice whereas those vaccinated under conditions of CDP blockade showed just 5% survival (Fig. 2g). Finally, we sought to understand whether the failure of DC vaccination was caused only by a decreased in total cell number, or also by an actual defect in differentiation due to CDP blockade. Mice adoptively transferred with a low number of OT-I cells were vaccinated and LFA-1-dependent interactions were blocked during the CDP. OT-I cells were isolated from those mice 6 days after vaccination with or without CDP blockade. An equal number of OT-I cells was then transferred in naive recipients to allow these to establish memory. Recipient mice were challenged with a lethal dose of LM-OVA at least 60 days after transfer. OT-I cells generated from DC vaccination in the context of CDP blockade were unable to effectively protect mice compared to cells arising from control vaccination ($P < 0.01$; Fig 2h). In summary, we concluded that not only cell number but also cell differentiation are regulated during the CDP and necessary for optimal DC vaccination.

Functional Role for cell interactions beyond T cell - APC

The synaptic requirement of LFA-1 on T cells has been linked with stabilized binding to antigen-presenting DC bearing the counter ligand ICAM-1²⁵. We therefore sought to formally establish the requirement for DCs as the synaptic partners of T cells during the CDP. To do so, mice were immunized with a pure population of antigen-pulsed BMDCs generated from CD11c-DTR mice²⁶. We injected diphtheria toxin (DT) in such a way that

the adopted APCs were fully ablated by the start of the CDP without affecting T cell priming (Supplementary Fig. 4). Both host and responding OT-I T cells were homozygous for the H-2^{bml} allele in our study, rendering them incapable of presenting peptides to the OT-I cells on their own MHC. APC ablation, which was complete by 24 hours post-immunization (Supplementary Fig. 4), did not significantly affect IFN- γ production by OT-I cells (Fig. 3a) or their expansion (Fig. 3b). However, CDP blockade 24 hours post-immunization in the context of APC ablation significantly inhibited both measures. We noted that previous studies have established that prolonged APC interaction does not control functionality of CD8⁺ T cell response *in vitro*^{27–29} and *in vivo*³⁰ but had suggested that T cell differentiation was cell autonomous post-APC encounter. Our data confirm these previous findings but strongly suggest that the differentiation cue during the CDP is, in fact, reliant on an adhesive interaction with another cell or surface.

It has been observed that T cells utilize LFA-1 to form homotypic 'clusters', forming a relatively transient T-T synapse^{9,31}. That interaction requires T cells to bear ICAM-1 (Fig. 3c, Supplementary Fig. 5a–b). However, ICAM-1 expression on T cells was not necessary for T-APC interaction since OT-I *Icam1*^{−/−} T were at least as proficient in forming stable interactions with antigen-bearing DCs (Supplementary Fig. 5c). We tested the requirement of ICAM-1 on T cell for effector differentiation by adopting varying numbers of allelically marked wild-type or *Icam1*^{−/−} T cells into the same hosts and measuring the percentage of cells expressing IFN- γ 6 days after immunization with DEC-OVA (Fig. 3d). Differentiation of the ICAM-1-deficient T cells was impaired compared to controls when 1000 cells of each type were transferred. Larger numbers of transferred cells rescued inhibition of CD8 differentiation induced by ICAM-1 deficiency. Taken together, we concluded that there is a requirement for T cells to be bound by other cells, likely other T cells, especially when physiologically relevant numbers of T cells were activating. Expression of full-length ICAM-1 on CD8 T cells was similarly required for optimal CD8 response against LM-OVA (Fig. 3e) and LCMV (Fig. 3f). Mice bearing ICAM-1-deficient OT-I T cells adoptively transferred at low numbers were also poorly protected in vaccination for protection against a lethal dose of *Listeria* compared to mice bearing the same number of wild-type OT-I T cells (Supplementary Fig. 5d). From those experiments, we concluded that ICAM-1 expression on T cells, and therefore cell interactions beyond those mediated by APC, are formative for CD8 T cell differentiation in response to immunization.

T-T interactions are autonomous but facilitated by APC

This finding led us to consider T cells themselves as an alternative LFA-1 bearing partner at this time, and so we examined the dynamics of T cell interactions centered on the CDP, in the presence or absence of ICAM-1 on T cells. We turned to 2-photon microscopy of lymph nodes using wild-type or *Icam1*^{−/−} cells labelled with distinct dyes, using 2×10⁶ adoptively transferred cells to facilitate statistical analysis. In time-projections of T cell zones, ICAM-1-deficient T cells were less stable in their positions at 24 hours compared to their wild-type counterparts but were in the same T cell compartments as each other (Fig. 4a and Supplementary Fig. 6a). Further, ICAM-1-deficient T cells typically left clusters more quickly: either when those clusters contained only ICAM-1-deficient cells or were a mix of wild-type and *Icam1*^{−/−} cells (Fig. 4b, Supplementary Movie 5). This finding suggests T

cells must be able to be bound to optimize arrest adjacent to other T cells. CDP blockade using LFA-1 Ab also decreased the percentages of OT-I in T-T clusters by approximately by half within 2 hours of blockade (Fig. 4c and Supplementary Movies 3,6) showing that T-T interactions are similarly inhibited by blocking either LFA-1 or its ligand.

ICAM-1 on T cells is thus required to stabilize T cell position adjacent to other T cells during the deceleration phase. Consistent with APCs being the primary nucleator of decelerated T cells, OT-I T cells moved faster and arrested for a shorter period when APCs were ablated during the CDP (Fig. 4d,e and Supplementary Movies 7,8). Under these conditions, T-T contacts were similarly stable over the first ~10 minutes, although T cells were then more weakly associated over longer times (Fig. 4f and Supplementary Movies 7,8). T-T contact stability with similar lifetime of association was previously observed for CD4⁺ T-T synapses⁹. Taken together, this indicates that arrest and interaction is a milieu affect, driven both by APC and via lateral homotypic interactions.

A secondary “collective” phase of cellular programming, during which primed T cells mingle, would require T-T interactions to be not only avid but also sufficiently frequent, particularly when the number of primed cells are limited. By surveying entire lymph nodes 24 hours post-immunization, we found that T-T contacts are selected for and occur when precursors are introduced at physiological frequencies (Fig. 4g and Supplementary Fig. 6b). Quantification of the recovered cells in lymph nodes demonstrated an immunization-dependent increase in the frequency of 2-, 3- and 4-cell containing clusters at this time (Fig. 4h). This validated that close T-T contacts are a feature of priming, even under low precursor frequencies. Although the mechanism for promoting the interaction may simply involve ongoing random migration and selective adhesion, it is also possible that these interactions profit from early chemokines to find one another at dynamically selected sites within lymph node volumes^{7,8}.

T-T Interactions Promote Critical Synaptic Cytokine Exchange

T cell differentiation is largely driven by cytokines and these can be directed into both T-APC and T-T synapses^{9,32}. CD8⁺ T cells begin to make IFN- γ within 24 hours upon immunization (Supplementary Fig. 7a and³³). By expressing IFN- γ fused to green fluorescent protein (GFP) in T cell blasts and tracking T-T contacts, we observed that vesicles containing IFN- γ are recruited to the site of contact (Fig. 5a,b and Supplementary Movie 9). Furthermore, T cells participating in clusters in the absence of APCs *in vitro* indeed secreted IFN- γ (Fig. 5c), and they did so preferentially inward toward one-another (Fig. 5d). IFN- γ was secreted at sites of T-T contact where ICAM-1 enrichment was also found (Supplementary Fig. 7b and Supplementary Movie 10), revealing the existence of an immunological synapse between CD8⁺ T cells. Intracellular IFN- γ was directed between adjacent T cells *in vivo* during the CDP upon immunization (Fig. 5e). To investigate the function of IFN- γ secretion from one T cell to another, we primed T cells in the absence of APCs, using pharmacological mimics of TCR signalling and blocked synaptic interaction via LFA1 Ab, in the presence or absence of exogenous IFN- γ or IFN- γ blockade. Cells were then transferred into mice for approximately 30 days and assayed in a recall response (Supplementary Fig.7c). Blockade of either LFA-1 or IFN- γ in the first day of APC-free

stimulation reduced the percentage of IFN- γ positive cells after recall (Fig. 5f). In this assay, *in vitro* treatment with LFA-1 Ab, but not IFN- γ Ab, also blocked overall recovery of T cells, which may be an effect on homing back into lymph nodes or may reflect the requirement for other signals delivered at T-T contacts (Fig. 5g). Specific add-back of IFN- γ cytokine to this assay resulted in a dose-dependent recovery of IFN- γ production by differentiated cells after recall (Fig. 5h), consistent with this signalling axis being sufficient as well as necessary for differentiation. However, full restoration in the presence of LFA-1 Ab required 50 times more than the concentration of IFN- γ typically utilized to skew differentiation when T-T contact is untouched (data not shown). For reasons that are at present unclear, very high IFN- γ doses also restored the number of cells recovered (Fig. 5i) even in the presence of LFA-1 Ab. The IFN- γ receptor CD119 was also required for CD8⁺ T cells at physiological precursor frequencies to commit to producing the cytokine *in vivo* (Supplementary Fig. 7d–g). Taken together, we concluded that IFN- γ shared through T-T synapses contributes to CD8 T cell differentiation.

Discussion

These results provide evidence of a second stage of information exchange through cell-cell communication, which is necessary for an effective immune response. We propose that during motility arrest, prolonged juxtaposition to APC in the T cell zone also facilitates other types of cell-cell synaptic communication, including T-T synapses, which enhance collective differentiation. A collective phase may involve additional cell types beyond T cells that join clusters and may also involve additional cytokines beyond those studied here.

Although it has previously been supposed that the arrest phase primarily mediated key interactions with APCs, previous evidence suggests that such prolonged APC interaction does not control functionality of CD8⁺ T cell responses *in vitro*^{27–29} and *in vivo*³⁰. Consistent with antigen-presentation being most relevant only at early time, when cell-cell interactions are blocked during the CDP, we did not inhibit CD69 up-regulation and proliferation. Motility arrest in the T cell zone therefore appears to have other functions than TCR triggering. Specifically T cell-T cell contacts during the CDP regulate the balance between effector and memory cells, but also potentiate CD8 cell amplification and/or survival. Blockade at 24 hours resulted in apparent defects already at day 6 and profoundly poor protection in the late phase. Analysis of markers show that the early defects corresponded to an early failure to commit to central memory cells, cells which are critical for lasting protection³⁴.

We describe that clustering of T cells can be found upon immunization when physiological precursor frequencies are used. But how do rare T cells converge on particular sites in the lymph node? Evidence exists showing DCs produce chemokines CCL3 and CCL4 that attract CCR5-expressing CD8⁺ T cells in a CD4⁺ T cell-dependent manner, and it had been proposed that this phenomenon guided antigen-specific CD8⁺ T cells to DCs for priming⁷. APCs, especially those that have been helped by CD4⁺ T cells, would produce such chemokines and assist in attracting experienced CD8⁺ T cells and bringing them together. This scenario may underlie the reason why T-T interactions may function effectively at low

precursor frequency and in this scenario, clustering and ensuing “collective” differentiation would be a consequence of T cell help.

T-T interactions were not recognized to play a part in end-point assays, although formation of T cell clusters following T cell activation has been documented as a nucleation around APCs and seen as a read-out of strong T cell activation^{9,12,15–17}. In the present study, we provide evidence that CD8⁺ T cells not only obtain information from the APC in these clusters, but also from the other T cells; both *in vitro* and *in vivo*. The exchange of information between CD8⁺ T cells requires integrin-mediated contact and formation of a T-T synapse. Although T-T synapse and cytokine sharing have been already described for CD4⁺ T cells *in vitro*⁹, we now demonstrate that CD8⁺ T cells also share cytokines, including IFN- γ , *in vitro* as well as *in vivo*, and more importantly that T-T communication is a relevant facilitator of the downstream output: namely T cell differentiation. T cell differentiation resulting from T-T adhesive ‘secondary’ synapses provide an alternative platform to the immune synapse for very local cytokine exchange. Such contact might also provide a platform to facilitate asymmetric cell division³⁵ rather than the APC.

While it is well established that IFN- γ is crucial for T_H1 differentiation³⁶, our results suggest that its importance for CD8⁺ T cell differentiation had been so far underestimated. One reason for this could be the frequency of Ag-specific CD8⁺ T cells used in previous studies, which we show are distinctly important, as other signals appear to be sufficient in our studies when a overly large number of Ag-specific CD8⁺ T cells is used. We speculate that in response to stimuli beyond the ones tested in our study, IFN- γ may also have a different impact on T cells, perhaps through its production or action on additional partners³⁷. While some cytokines in some responses are certainly dispersed globally^{9, 38}, secondary synapse and localized synapse provide specificity as well as amplification, likely even under those conditions. Finally, our work suggests a careful exploration of cytokine receptor aggregation within synapses since LFA-1 blockade reduced sensitivity to cytokine delivered at T-T contacts. Indeed, the expression and activity of IFN- γ R1, CD119, is regulated upon immunization^{39,40}, and one can hypothesize that this may actually lead to a heightened selection for cell-cell delivered cytokine.

Our data suggest that T-T contacts enhance CD8⁺ T cell expansion and differentiation. However, we propose that direct communication between T cells allow them to collectively respond and control the size of the effector and memory pool. This implies that while some cells would get rescued or amplified, others would be deleted. This could be true especially for a polyclonal response, and T cell clusters would be more typically composed of heterogeneous T cells that influence each other's fate. This would explain how heterogeneous T cell populations respond in a coordinated manner.

T-T synapses may facilitate and underlie other exchanges of information besides cytokines. For example, T cells have been shown in some experiments to capture peptide-MHC complexes and mediate Ag-specific signalling to other CD8 (ref. ⁴¹). Similarly, Ag-specific CD4⁺ T-T interactions may regulate cell expansion following CD4⁺ T cell up-regulation of MHCII expression⁴². Finally, a recent study suggested that cells that up-regulated the Hippo

pathway may commit to terminally effector differentiation⁴³ and the synapse process we characterize may provide a framework for these.

Collective behaviour typically arises when a collection of organisms or cells coordinate their responses. For instance, colonies of bees make a collective decision to select the best nectar source not through individuals visiting all sources but by later comparisons at the hive⁴⁴. At a cellular level, in collective germ-cell migration, each cell can move within the cluster and function somewhat autonomously but yet collection of cells migrates toward a stimulus⁴⁵. Similar behaviour has been observed during cancer metastasis where cooperation between invasive and non-invasive cells enables extravasation of otherwise non-metastatic cells⁴⁶. Collective decision-making is thus a collection of stochastic events that, through positive reinforcement, allows the individual components to select the optimal response for the system. The immune system represents, perhaps, a novel twist on this as it uses the rather transient formation of synapses between many cell types to achieve the goal of collective decisions. The formation of T-T synapses would appear in this context to provide a feedback system to compare and select an effector/memory response dictated by the experiences of other individual activated T cells. Notably, these types of interactions, hours after critical T-APC interactions initiate T cell activation, may provide feedback that regulates many other facets of the response, including system-wide 'tolerance'. More broadly, immune synapses between many different types of immune cells, and not just between T cells and APCs, may represent a critical mechanism to enhance collective decision-making, and at the same time to also limit the exposure of adjacent cells to effector signals. This work thus establishes a framework for considering synapses as being mediators that integrate information across many concurrently activating cells and generates concerted immune responses.

Methods

Mice

Icam1^{-/-} mice (The Jackson Laboratory) were crossed with ovalbumin (OVA)-specific TCR-transgenic OTI CD45.1^{+/+} to generate OTI CD45.1^{+/+} *Icam1*^{-/-} mice, and were crossed with gp33-41 LCMV peptide-specific transgenic P14 CD45.1^{+/+} to generate P14 CD45.1^{+/+} *Icam1*^{-/-} H2b^{bm1} mice (The Jackson Laboratory) were crossed with OTI CD45.1^{+/+} to get OTI CD45.1^{+/+} H2b^{bm1} mice. These mice, C57BL/6 (The Jackson Laboratory and Simonsen), CD11c-DTR, Yet40 mice, H2b^{bm1}, CD11c-YFP, *Ifngr*^{-/-}, P14 CD45.1^{+/+}, OTI CD2-RFP and OTI CD45.1^{+/+} mice were housed and bred under specific pathogen-free conditions at the University of California Animal Barrier Facility. All experiments involving mice were approved by the Institutional Animal Care and Use Committee of the University of California.

Cell isolation

OTI or P14 T cells were isolated from lymph nodes and spleen of 6 to 12 week-old mice. Selection was carried out using a negative CD8 isolation kit (STEMCELL Technologies Inc.). Bone Marrow-Derived Dendritic Cells (BMDCs) were generated by culturing bone marrow cells for 8–11 days with GM-CSF. IL-4 was added for the last 2 days of culture.

Cell transfer, immunization, anti-LFA-1 treatment and recall

Where indicated, OTI or P14 cells were labelled with 2 μ M of cell proliferation dye CFSE (Invitrogen) for 30 min at 37°C and transferred in recipient mice using retro-orbital injection. Mice were immunized 16 h later. For Anti-DEC205–OVA (NLDC-145) (DEC-OVA) immunization, the indicated dose of DEC-OVA conjugates (produced in house) was injected subcutaneously (s.c) in both flanks in the presence of 10 μ g CD40 Ab (1C10, eBiosciences). For LM-OVA immunization, intravenous injection (i.v) of 10×10^3 colony-forming units (CFU) of *L. monocytogenes* that expresses a secreted form of OVA (LM-OVA)⁴⁷ was performed. For LCMV immunization, i.v injection of with 1×10^6 PFU LCMV Armstrong was performed. For DC ablation experiments, BMDCs were generated from CD11c-DTR mice and pulsed with 30 ng/ml of the ovalbumin peptide SL8 (SIINFEKL) (AnaSpec) for 30–60 min at 37 °C. Mice were immunized with s.c injection of 1×10^5 CD11c-DTR BMDCs per flank, in the presence of 200 ng/ml LPS in PBS. At the indicated time after immunization, DT (200 ng/animal) (Sigma) was administered by intra-peritoneal (i.p) injection.

In some experiments, mice received 100–200 μ g of either isotype control (RatIgG2a, BioXCell) or anti-LFA-1 (M17.4, BioXCell) every 12 h for 36 h, as indicated. For blocking of homing and synchronizing T cell activation, mice were treated with 200 μ g of CD62L Ab (Mel-14) when indicated. For recall experiments, mice were re-challenged s.c with 0.2 μ g DEC-OVA and 10 μ g CD40 Ab, 28 to 30 days after the primary immunization.

DC vaccination and challenge with LM-OVA

Mice were transferred with 5×10^3 WT or *Icam1*^{−/−} OTI cells and vaccinated with 2×10^4 SL8-pulsed BMDCs on both flanks in the presence of 200 ng/ml LPS. When indicated, mice received LFA-1 Ab treatment as described above. In some experiments, OTI cells were isolated 6 days post-vaccination and transferred back in naïve recipients. 40–70 days post-vaccination, mice were challenge with a lethal dose of LM-OVA ($2-10 \times \text{LD}_{50}$).

Surface and intracellular flow cytometry staining

Cells were washed in PBS, and blocked in flow cytometry buffer (PBS, 2% FCS, 2 mM EDTA, 0.1% sodium azide) containing CD16/32 Ab (2.4G2). Staining for surface proteins with conjugated-antibodies against CD45.1 (A20), CD45.2 (104), CD8 (53.6.72), CD62L (Mel-14), CD44 (IM7), CD69 (H1.2F3), KLRG1 (2F1) (eBiosciences) and IL-7R (B12-1) (Biolegend) was performed in FACS Buffer for 20 min at 4°C. Cells were washed and resuspended in flow cytometry buffer containing 1% PFA.

For intracellular cytokine staining, mice were sacrificed 5 to 6 days post-immunization or 4 days post-recall. Lymph node cells were re-stimulated *ex vivo* for 4 h with 100 ng/ml SL8 peptide or 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 3 μ g/ml BrefeldinA (Sigma-Aldrich) for the last 2 h. Cells were stained for surface proteins and fixed in flow cytometry buffer 2% PFA. Cells were then permeabilized with flow cytometry buffer containing 2% Saponin for 5 min, and stained with conjugated-IFN- γ Ab (XMGI.2, eBiosciences) in flow cytometry buffer 1% Saponin for 15 min at 20°C. Cells were kept in flow cytometry buffer 1% PFA prior to analysis.

Quantification of endogenous OVA-specific CD8 cells

Lymph node cells were stained with R-PE conjugated MHC-I pentamer specific for the OVA peptide SIINFEKL (Proimmune) in flow cytometry buffer. Subsequently, cells were stained for CD8 and analyzed by flow cytometry.

In vitro T cell priming

Nave OTI cells were plated at low density (0.5×10^6 cells/5ml) activated with 2 ng/ml PMA and 20 ng/ml ionomycin. Cells were treated, when indicated, with 20 μ g/ml LFA-1 Ab (M17.4), 20 μ g/ml IFN- γ Ab (XMG1.2, BioXCell), 10 ng/ml or 500 ng/ml IFN- γ (Peprotech). Where indicated, two to three days after priming, cells were transferred by retro-orbital injection into WT recipients.

In vitro T-DC coupling assay and T-T clustering assay

BMDCs were matured with 1 μ g/ml LPS 1 day before and pulsed with 100 ng/ml SL8 peptide for 30 min. BMDCs and nave OTI cells were labelled with 4 μ M DDAO (Invitrogen) and 1 μ M CFSE, respectively. Flow cytometry-based coupling was analyzed as described⁴⁸.

For T-T clustering, nave WT and *Icam1*^{-/-} OTI cells were labelled with 1 μ M CFSE and 2 μ M CMTMR (Invitrogen), respectively. Cells were ad-mixed, and activated with 5 ng/ml PMA and 50 ng/ml ionomycin. After 24 h, cells were fixed in 2% PFA, and were analyzed by microscopy or flow cytometry. For flow cytometry, cells were run through a 40 μ m strainer to separate clustered from non-clustered cells.

IFN- γ -GFP expression in T cell blasts

Mouse IFN- γ was fused to the GFP using the restriction sites XhoI and AgeI. Then the cDNA of this fusion protein was inserted in MSCV (pBabe MCS-IRES-RFP; Addgene) using the restriction sites XhoI and NotI. Nave OTI cells were activated with 2 μ g/ml plate-bound CD3 Ab (2C11) and 2 μ g/ml CD28 Ab (PV1). Plasmid encoding for IFN- γ -GFP was transfected in the phoenix packaging cell line using calcium phosphate. Virus-containing supernatant from these cells was used on two consecutive days (days 2 and 3 after activation) to spin-infect T cell blasts. Transduced cells were used 4 days after activation.

IFN- γ capture assay and confocal microscopy

OTI cells were coated with mouse IFN- γ catch reagent from a mouse IFN- γ secretion assay detection kit (Miltenyi Biotec) and activated with 5 ng/ml PMA and 50 ng/ml ionomycin on Fibronectin-coated chambers. After 24 h, cells were fixed with 1% PFA for 15 min at 4 C, and stained with PE- or APC-conjugated IFN- γ detection antibody. Cells were analyzed with an inverted Zeiss with Yokogawa CSU-10 Spinning Disk. The imaging and control software used was MetaMorph (MDS Analytical Technologies).

Histology

For cell clustering at low precursor frequency, serial 60- μ m sections of PFA-fixed and frozen pLNs were incubated in cold acetone for 20 min. Total number of CFSE-labelled or

GFP OTI and frequency of clusters were quantified for the whole LN. For IFN- γ staining, 20- μ m sections of PFA-fixed and frozen LNs were stained with anti-IFN- γ and Alexa488-conjugated anti-GFP (invitrogen). Sections were then washed and incubated with Cy5- or Rhodamine-conjugated anti-Rat. All sections were analyzed by confocal microscopy.

2-Photon imaging of explanted lymph nodes

To study T cell clustering kinetics, OTI-RFP (3×10^6) cells were transferred to CD11c-YFP recipients. To study T cell clustering ability of *Icam1*^{-/-} OTI, WT and *Icam1*^{-/-} OTI cells were labelled with 2 μ M CFSE and 20 μ M CMTMR, respectively, ad-mixed and transferred to WT recipient. Switching dyes did not affect results (data not shown). Mice were immunized s.c. in footpads and flanks with 2 μ g DEC-OVA and 10 μ g CD40 Ab or were left unimmunized as a control. When indicated, 150 μ g of LFA-1 Ab was administered s.c. To study T cell behaviour after BMDCs ablation, OTI H2b^{bm1} cells were labelled with 2 μ M CFSE and transferred into H2b^{bm1} mice. Mice were then immunized with CMTMR-labelled and SL8-pulsed BMDCs generated from Cd11c-DTR mice. When indicated, mice were treated with diphtheria toxin 8 h post-immunization. Draining LNs were taken out when indicated and immobilized on coverslips with the hilum facing away from the objective.

Time-lapse imaging was performed with a custom resonant-scanning instrument containing a four-photomultiplier tube (Hamamatsu) operating at video rate, as described²⁰. Each *xy* plane spanned 288 μ m $\times$ 240 μ m at a resolution of 0.60 μ m per pixel. Images of up to 35 *xy* planes with 3- μ m *z*-spacing were acquired every 30 s for 30 min.

Imaris (Bitplane) and Matlab software (Mathworks) were used to quantify T cell speed and cell clustering. T-T interaction was defined as the close association of a given OTI cell with another OTI cell for at least 3 min. A threshold of 4 μ m between cell edges was used, which account for low fluorescence frequently encountered at cell edges, and fits manual quantification (data not shown).

Statistical analysis

Data were expressed as mean $\pm$ SEM. Comparisons between groups were analyzed with the *t*-test, one-way or two-way Anova test, using GraphPrism software. Data were considered as statistically significant when *P*-values were ≤ 0.05 .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Temporal requirement for CD8 T cell differentiation

(a) CD11c-YFP mice were adoptively transferred with OT-I-RFP cells. Individual “snapshots” of OTI cells (red) and DCs (green) acquired 2, 10, 24 or 72 hours post-immunization with DEC-OVA. Images are displayed as maximum intensity projections along the z axis (top view). Lower panels: Pseudo-colored time projection of a 30 minutes run showing the spatial persistence of OTI cells in clusters 24 hours, but not 2, 10 hours and 72 hours after immunization. Image intensities were scaled to a normalized time projection intensity range of 0–1. Scale bar, 30 μ m. Data representative of at least three independent experiments (b) T cell priming (CD69 blue label) was quantified by CD69 expression on OTI cells 32 hours post-immunization with DEC-OVA with temporal LFA-1 blockade. T cell differentiation (IFN- γ yellow label) was quantified as the percentage of OTI secreting IFN- γ 6 days after immunization with temporal LFA-1 blockade. Results are expressed as percent of induction compared to immunization without temporal blockade. $n=5$ - graphs indicate the percentage of induction compared to the control mice – error bars represent SEM (c,d) Influence of temporal LFA-1 blockade on the percentage of OTI among CD8 (c, $*P < 0.05$) and percentage of OTI secreting IFN- γ (d, $*P < 0.001$) after LM-OVA immunization. Data are from three independent experiments. Each dot is an individual mouse. (e,f) Influence of temporal LFA-1 blockade on the percentage of P14 among CD8 (e, $*P < 0.05$) and percentage of P14 secreting IFN- γ (f, $*P < 0.05$) after LCMV immunization. Data are from three independent experiments. Each dot is an individual mouse. (g–h) Influence of temporal LFA-1 blockade on the endogenous response against DEC-OVA in YETI mice as quantified by the percentage of pentamer positive CD8 cells (g, $*P < 0.01$) and percentage of YFP positive pentamer cells (h, $*P < 0.01$). Data are from four independent experiments. Each dot is an individual mouse.

Figure 2. Generation of central memory precursor cells and recall response are dependent on LFA-1-dependent stable interactions during the CDP

(a–c) Mice adoptively transferred with 5×10^3 P14 cells were immunized with LCMV and LFA-1 was blocked during the CDP. 15 days post-immunization, the number of P14 cells (a, * $P < 0.05$) and the expression of surface markers KLRG1, IL7R (b, * $P < 0.05$ and ** $P < 0.001$) and CD62L (c, * $P < 0.01$) were analyzed. SLEC population was defined as KLRG1 high, IL7R low P14 cells, and MPEC population was defined as KLRG1 low, IL7R high P14 cells. Data are from three independent experiments - $n = 6$ – error bars correspond to SEM. (d–f) Mice adoptively transferred with 5×10^3 OTI cells were immunized with DEC-OVA and LFA-1 was blocked during the CDP. d- Percentage of CD44 and CD62L positive cells among OTI cells was analyzed 8 days post-immunization. * $P < 0.001$. Data are from three independent experiments. Percentage of OTI secreting IFN- γ (e, * $P < 0.05$) and percentage of OTI among CD8 (f, * $P < 0.001$) were quantified after recall with low dose of DEC-OVA 30 days post-immunization. Data are from three independent experiments. Each dot is an individual mouse.

(g–h) Mice adoptively transferred with 5×10^3 OTI cells were vaccinated with OVA peptide-pulsed DCs and LFA-1-dependent interactions were blocked during the CDP. (g) Forty to sixty days after vaccination, mice were challenged with a lethal dose of LM-OVA ($2-10 \times$ LD50). $P < 0.001$ - $n = 13$ (h) Six days post-immunization, OTI cells were isolated and the same number of cells was transferred back in naïve mice. Seventy days after vaccination, mice were challenged with a lethal dose of LM-OVA ($2 \times$ LD50). $P < 0.01$ - $n = 10$ - Data are from two independent experiments.

Figure 3. CD8 cell differentiation largely relies on T-T contacts

(a–b) H2b^{bm1} mice bearing H2b^{bm1} OTI cells were immunized with OVA peptide-pulsed BMDCs generated from CD11c-DTR mice. APCs were ablated (DT) or LFA-1-dependent interactions were inhibited (CDP blockade) during the CDP. Percentage of OTI secreting IFN- γ (a, * $P < 0.001$) and percentage of OTI among CD8 (b, * $P < 0.05$) were analyzed at the peak of the effector response. Data are from at least three independent experiments. Each dot is an individual mouse. (c) 1×10^6 fluorescently labelled WT (green) and ICAM-1 $^{-/-}$ (red) T cells were ad-mixed in a flat-bottomed well and activated with PMA+Ionomycin. 24h post-activation, the presence of WT vs ICAM-1 $^{-/-}$ cells in clusters was evaluated by microscopy. Scale bar, 5 μ m. (d) The effector response of WT and ICAM-1 $^{-/-}$ OTI cells after DEC-OVA immunization was quantified as the percentage of OTI secreting IFN- γ 6 days post-immunization. * $P < 0.05$ and ** $P < 0.001$. Data are from three independent experiments. (e) The effector response of WT and ICAM-1 $^{-/-}$ OTI cells after LM-OVA immunization was quantified as the percentage of OTI secreting IFN- γ 8 days post-immunization. * $P < 0.01$. Data are from three independent experiments. (f) The effector response of WT and ICAM-1 $^{-/-}$ P14 cells after LCMV immunization was quantified as the percentage of P14 secreting IFN- γ 8 days post-immunization. * $P < 0.05$. Data are from three independent experiments.

Figure 4. Characterization of T-T contacts

(a–b) Fluorescently labelled WT and ICAM-1^{-/-} OTI cells (2×10^6) were transferred in WT recipients. Mice were immunized with DEC-OVA. **a**- Individual “snapshot” showing WT (green) and ICAM-1^{-/-} (red) OTI cells during the CDP, i.e 24h after immunization *in vivo*. Images are displayed as maximum intensity projections along the z axis (top view). Image intensities were scaled to a normalized time projection intensity range of 0–1. Lower panels: Pseudo-colored time projection of a 30 minute run showing the spatial persistence of WT (left panel) and ICAM-1^{-/-} (right panel) OTI cells. Scale bar, 15μm. **b**- Graph shows the fraction of OTI cells remaining in cluster over time *in vivo* after DEC-OVA immunization. $P < 0.001$. Data are from two independent experiments.

c- CD11c-YFP mice bearing OTI-RFP cells were immunized with DEC-OVA. When indicated, mice were treated with LFA-1 Ab 22h post-immunization. Explanted lymph nodes were subjected to 2-photon imaging 2h after LFA-1 Ab treatment. Graph shows the percentage of OTI cells engaged in homotypic interaction during a 30 minute run. Every dot corresponds to an individual field. $*P < 0.001$. Data are from four independent experiments.

(d–f) H2b^{bm1} mice bearing CFSE-labelled OTI H2b^{bm1} were immunized with CMTMR-labelled and OVA peptide-pulsed BMDCs generated from Cd11c-DTR mice. When indicated, mice were treated with DT 8h post-immunization (DT). Explanted lymph nodes were subjected to 2-photon imaging 24 to 30h post-immunization. Instantaneous speed **(d)**, Arrest Coefficient (defined as the percentage of time a given cell has an instantaneous speed $< 2 \mu\text{m}/\text{min}$, every dot correspond to an individual cell) **(e)**, $*P < 0.001$, and the fraction of OTI cells remaining in cluster over time **(f)** were analyzed during the CDP. **g**- Example of a cluster composed of 4 OTI cells found during the CDP after immunization with DEC-OVA

when low precursor frequency (10^4 OTI cells transferred) is used. Image is displayed as maximum intensity projection along the z axis (top view). Cells are numbered from 0 to 4. Cell #0 is isolated, whereas cells #1 to 4 are clustered. **h-** Quantification of the percentage of OTI cell clusters found in a whole popliteal LN of naïve or immunized animals when low precursor frequency is used (n=338 for naïve, n=320 for DEC-OVA condition, over 3 independent experiments). *P < 0.05 and **P < 0.001.

Figure 5. OTI cell differentiation is regulated by cytokine secretion at T-T contacts (a–b) OTI blasts were transduced with a plasmid encoding IFN- γ fused to GFP (IFN- γ -GFP). Cells were stimulated with PMA and Ionomycin to induce cell clustering and imaged for 15 minutes. Time-lapse images in (a) show an IFN- γ -GFP expressing OTI cell at the periphery of a cluster. Upper panels: Overlay between IFN- γ -GFP and contrast. Scale bar, 10 μ m. Lower panel: magnification showing IFN- γ -GFP localization within a cell (cell contour is represented by a dashed gray line) relative to cell-cell contact (depicted in red). Scale bar, 5 μ m. Graph in (b) shows the percentage of IFN- γ -GFP expressing OTI cells at the periphery of a cell cluster showing localization of IFN- γ -GFP at T-T contact, or not specifically localized (n=20). *P<0.01. (c–d) OTI cells were stimulated with PMA +Ionomycin for 24 hours. Representative images of the sites of IFN- γ secretion in clustered cells (c, Scale bar, 10 μ m) and quantification of percentage of cells at the periphery of a cluster showing captured IFN- γ facing another T cell or facing away (n=50) (d, *P<0.05). Data are from three independent experiments. e- Picture shows an example of 2 OTI clustered cells with polarized IFN- γ localization *in vivo* during the CDP after DEC-OVA immunization. Cells are shown in the xyz dimensions. OTI cells are green and IFN- γ is depicted in red. Scale bar, 5 μ m. f- Time-line of the experimental protocol. (f–i) OTI cells were activated in vitro with PMA and Ionomycin, treated with LFA-1 Ab, IFN- γ Ab (f–g) or IFN- γ (h–i) and transferred in WT mice 3 days post-activation. Percentage of OTI secreting IFN- γ (f and h, * P<0.01 **P<0.001) and percentage of OTI among CD8 (h and j, *P<0.05 and **P<0.001) were analyzed after in vivo recall. Data are from three independent experiments.