Targeting the tumor-draining area : local immunotherapy and its effect on the systemic T cell response

Herbert-Fransen, M.F.

Chapter 4

Separate roles for antigen recognition and lymph node inflammation in CD8+ memory T-cell formation.

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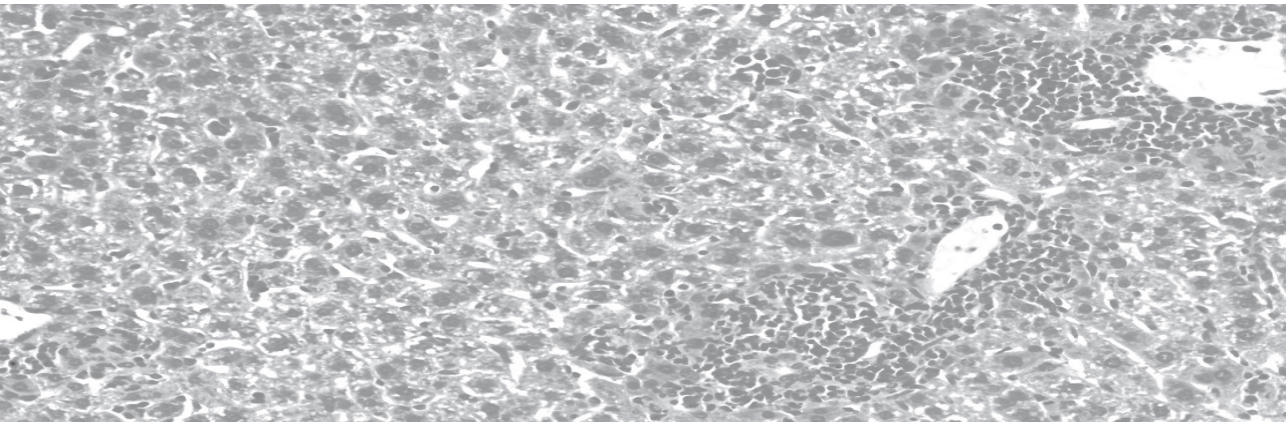

Abstract

Priming of naive CD8+ T-cells by pathogens or vaccines generally involves their interaction with antigen-loaded dendritic cells (DCs) in the context of an inflamed lymph node. Lymph node activation fosters DC and T-cell encounter, and subsequently provides newly primed T-cells with nurturing conditions. We dissected these two aspects by infusing in vitro primed CD8+ T-cells into naïve recipient mice harboring a single activated lymph node and comparing the fate of these T-cells with those infused into control recipients. Brief (20 hr) in vitro priming empowered the T-cells to expand in vivo without further antigen stimulation. This primary response was not affected by the presence or absence of a non-specifically activated lymph node. In contrast, in vivo antigenic challenge after contraction of the primary response resulted in significantly stronger secondary T-cell responses in mice harboring activated lymph nodes, demonstrating that the availability of an activated lymph node supported the generation of T-cell memory in an antigen unrelated manner. The presence of an activated lymph node during the expansion and contraction phase of the primary response did not endow T-cells with an instructional program for increased survival or secondary expansion, but primarily served to conserve increased numbers of T-cells.

Introduction

The initiation of T-cell responses upon primary encounter of pathogens involves the delivery of antigen and pathogen-associated molecular patterns (PAMPs) to secondary lymphoid organs draining the infected areas of the body. As a result, immunogenic peptide antigens are presented in MHC molecules at the surface of dendritic cells (DCs). Furthermore, the PAMPs trigger a sequence of events that orchestrate effective interaction between antigen-loaded DCs and naïve T-cells, including the expression of costimulatory molecules at the DC surface and the production of pro-inflammatory cytokines and chemokines that recruit and arouse a variety of immune cells. (1) The latter process causes lymph nodes to develop a state of inflammation and swelling, also referred to as lymph node congestion or activation(2). Chemokines like CCL9 and CCL21, cytokines including TNF-alpha and IFN-α/ß, and the chemo-attractant receptor S1P all play an important role in enhancing the cellularity of the lymph node during an immune response, thereby creating an inflammatory micro-environment supportive of T-cell priming.(3-8) The significance of PAMPs such as Toll-like receptor ligands in the induction of T-cell immunity has been demonstrated in numerous experimental models, showing that delivery of antigen without PAMPs as immune-adjuvants results in T-cell tolerance. Even though the importance of PAMPs for inducing costimulatory signals through DC activation as well as for triggering lymph node activation is broadly recognized, and the nature of the DC costimulatory signals has been studied in great detail (9,10), much less is known about the impact of lymph node activation on the effector and memory phases of the T-cell response. In the present study, we have focused on the latter aspect by separating DC-T cell engagement and lymph node activation in place and time. Our experimental data reveal that the availability of an inflamed lymph node during the primary response increases the magnitude of the secondary response through the conservation of larger numbers of T-cells.

Material and Methods:

Mice:

C57BL/6 jico (B6) mice were obtained from Jackson laboratories. OT-I TCR Tg RAG-1$^{-/-}$ mice were from W. R. Heath (The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia) and were bred in the animal facility of LUMC.v The experiments were approved by the animal experimental committee of Leiden University Medical Center.

Cell culture:

All in vitro cell culture and assays were performed in IMDM (Invitrogen Life

Technologies, Rockville, MD) supplemented with 8% v/v FBS (Greiner), 50 μM 2-ME, 2 mM glutamine, 100 IU/ml penicillin (complete medium). D1 cell line, a long-term growth factor-dependent immature splenic DC line derived from B6 mice, was kindly provided by P. Ricciardi-Castagnoli (University of Milano-Bicocca, Milan, Italy) and cultured as described (11). BM-derived DC were isolated from C57BL/6 jico femurs, cultured for 10 days in the same manner as D1 cells, before activation. The engineered APC cell line MEC.B7.SigOVA (SAMBOK) was generated as described (12), briefly, the mouse embryonic fibroblast cell line MEC-1 was transfected with CD80 and the minigene SigOVA, which encodes the $OVA_{257-264}$ (SIINFEKL) peptide directed to the ER, leading to efficient loading of the peptide onto MHC Class I molecules K^b. For in vitro priming, engineered APC were cultured at 70000 cells/well in 24 well plates overnight. The next day, nonadherent cells and cell debris were removed by washing the wells twice. OT-I cells were then added to the wells (0.5×10^6/well) in 2 ml of medium and plates were centrifuged for 1 min at 1000 rpm to initiate cell contacts. After 20h of co-culture the nonadherent OT-I cells were gently harvested, washed and adoptively transferred into recipient mice.

Lymph node activation:
D1 cells were cultured in D1 medium, maturation was induced by adding 6 μg/ml LPS (E-coli derived, serotype 026, B6, Sigma) 24 hours before injection. Cells were harvested with EDTA, washed three times with PBS, and injected, 2×10^6 in 30 μl PBS into the right hind leg footpad of mice.

In vivo challenge:
50 μg of synthetic short OVA peptide (SIINFEKL) was injected with 15 μg CpG 1826 (synthesized in the Leiden Institute of Chemistry) in 30 μl PBS into the right hind leg footpad of mice.

Flowcytometry:
All antibodies were purchased at BD and eBioscience. Intracellular cytokine staining was performed with the Cytofix/Cytoperm kit (Becton Dickinson) according to manufacturer's instructions.

In vitro proliferation assay;
Lymph nodes from mice were isolated from recipient mice and single cells suspensions were generated by mincing through cell strainers. Subsequently, cells were stained with CD8 and CD45.1 mAbs, and OT-I cells were purified by flowcytometric sorting. Equal amounts of cells were co-cultured with 100.000 irradiated spleen cells, and 1 μg/ml SIINFEKL, or medium control. 36 hours

later, cells were pulsed with 1 μC $_3$H per well, and analyzed 24 hours later.

Virus-infection:
Mice were infected with 5× 10^4 pfu influenza A/WSN/33 (WSN)-OVA_I (13) through intra-tracheal inoculation. After 5 days mice were sacrificed, and lungs were isolated in TRizol (Gibco) and homogenized. RNA was extracted and purified using the Qiagen RNeasy kit, cDNA was synthesized using 7.5 microgram RNA of each sample, and 12.5% cDNA was used in the RT-PCR reaction, in triplicate, and correlated to actin expression (primer/probe Applied Biosystems). cDNA synthesis and RT-PCR were performed using primer-sequences as described before (14)

Results
Activated lymph nodes provide increased storage for antigen-experienced T-cells
Lymph node activation is an intrinsic aspect of T-cell activation by infectious pathogens and vaccines. We separated the T-cell priming event from lymph node activation in space and time by exploiting an experimental model consisting of TCR-transgenic OT-I CD8+ T-cells and engineered APCs expressing high levels of the cognate ovalbumin (OVA)-derived peptide epitope and the costimulatory ligand CD80. Prior work had demonstrated that a 20-hr in vitro encounter with these APCs empowered naïve OT-I cells to vigorously proliferate in vivo upon transfer to naïve syngeneic recipient (15) Furthermore, these T-cells were shown to develop into long lived memory cells, capable of clonal expansion and protective effector function in response to secondary antigen encounter (16). Importantly, progression of the 20-hr primed OT-I T-cells through primary expansion, contraction and memory phases required neither in vivo exposure to antigen, nor the context of an inflamed, congested lymph node. This model therefore offered a unique opportunity to compare the in vivo behavior of primed CD8+ T-cells in the absence versus presence of a non-specifically activated lymph node. Activation of a single, popliteal lymph node in recipient mice was induced by footpad injection of activated syngeneic DCs (D1 cells or isolated BM-DCs) that were not loaded with antigen. Work by others has shown that this results in inflammation of the targeted lymph node within 1 day (8). The use of DCs not loaded with OVA antigen enabled us to separate the impact of antigen exposure and lymph node congestion in time and space. Comparison of the behavior of 20-hr ex vivo primed OT-I cells upon injection into DC-injected versus control mice revealed very few differences in the kinetics and magnitude of the primary response.

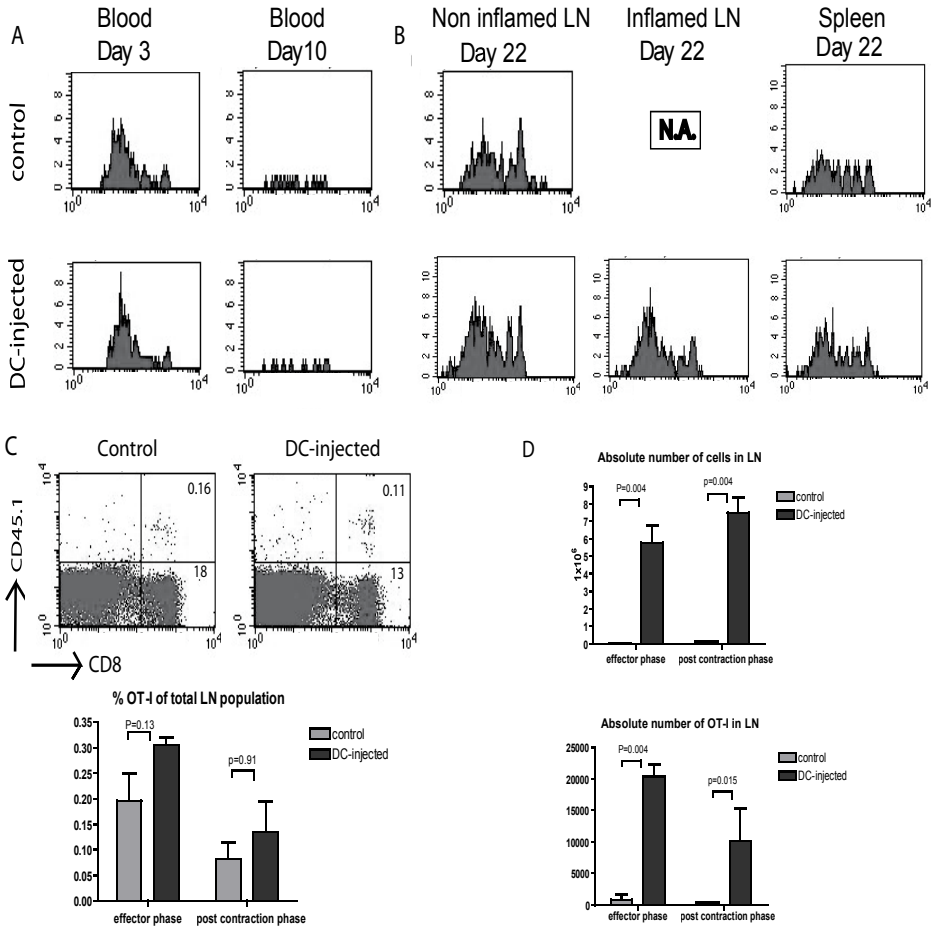

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Figure 1: Kinetics and magnitude of the primary in vivo response by 20 h in vitro primed OT-I cells.
CFSE-labeled naïve CD45.1 OT-I cells were cultured during 20 hrs with engineered APCs expressing OVA antigen and CD80, after which T-cells were separated from the APCs and injected into antigen-naïve B6 mice (1 ×106 per mice) as described previously (15). Recipient CD45.2 mice either did not receive prior treatment, or were injected into the footpad of the right hind leg with 2 × 106 in vitro, LPS-activated DCs (D1 cells), not loaded with antigen, 24 hrs prior to T-cell infusion . Blood samples were isolated at days 3 and 10 after start of T-cell activation. At day 22, mice were sacrificed and spleen and lymph nodes isolated. Panels A/B and C/D were from subsequent experiments with similar outcome. OT-I cells were gated by CD8 and CD45.1 A. Representative examples of groups of 4 mice (A, B) of proliferative response (CFSE) and relative OT-I cell counts in blood of DC-injected and control mice. B. Accumulation of memory OT-I cells during the post contraction phase in popliteal lymph nodes and spleen of DC-injected and control mice. Right-hand lymph nodes of DC-injected mice were inflamed. Contra-lateral, left-hand lymph nodes from DC-injected mice served as internal control. N.A. = not applicable. Three independent experiments were performed (A and B) C. Percentage of OT-I cells, in relation to total cell counts, in congested, right-hand lymph nodes of DC injected mice and the same lymph nodes from control mice in post contraction phase. Comparison revealed no difference in both effector and post contraction phase. D. Absolute total cell count and OT-I T cell count in congested, right-hand lymph nodes of DC injected mice and the same lymph nodes from control mice. Mann-Whitney test revealed significant differences in total and OT-I numbers in effector phase as well as in memory phase, 5 mice per group, two experiments pooled. Four independent experiments performed in total (C and D)

In both cases, the percentage of circulating OT-I cells peaked at day 3 after transfer, followed by a rapid contraction due to the absence of cognate antigen (Fig 1A). Three weeks after transfer, at a time when OT-I numbers in the blood were below detection, memory T-cells could still be detected in the spleen and lymph nodes (Fig 1B). As observed in the primary phase, percentages of memory OT-I cells differed neither between DC-injected and control mice (Fig 1C). However, the absolute total cell count was increased in the inflamed lymph nodes compared to contra-lateral lymph nodes in DC–injected mice and lymph nodes in control mice. Consequently, also the absolute numbers of OT-I cells were amplified in these lymph nodes, both at the peak of the effector phase and during the post-contraction phase of the primary response (Fig 1D). For inflamed lymph nodes, the total cell count was even larger in the post-contraction phase (day 22) than during the effector phase, in line with the notion that the size of the inflamed lymph nodes gradually increases and remains fully enlarged over at least 3 weeks. (data not shown). Taken together, our data suggest that the primary role of an inflamed, enlarged lymph node is to provide an expandable storage reservoir for memory T-cells.

Memory T-cell storage is important for the magnitude of the secondary T-cell response

Our initial observations prompted us to test how accumulation of greater numbers of memory T-cells in a single inflamed lymph node would impact on the systemic CD8+ T-cell response upon secondary antigen encounter. We delivered a secondary antigenic challenge to the in vitro primed OT-I cells through injection of ovalbumin peptide and CpG ODN into the footpad of the right hind leg, the same footpad into which mice with inflamed lymph nodes received their initial DC injection. In line with the greater number of memory T-cells available in these LNs in DC-injected mice, secondary responses in these mice were stronger than in control mice (Fig 2A, B). Moreover, the secondary responses in DC-injected mice were stronger than as compared to the primary responses recorded in these same mice, reminiscent of a textbook example of the relative magnitude of primary and secondary T-cell responses. Differences between DC-injected and control mice were most prominent in the blood and lung (Fig 2B), illustrating that secondary challenge of the DC-injected mice resulted in increased frequencies of effector T-cells capable of leaving the lymphoid organs and migrating to potential target tissues. Differences in OT-I T-cell frequencies were the least prominent in the draining lymph nodes, in line with this migratory behavior of effector T-cells and the increased overall cellularity of these lymph nodes (Fig 1D). Similar results were obtained in mice with an activated lymph node induced by injecting

bone-marrow derived DC. (data not shown).
Interestingly, the secondary responses in control mice did not exceed the magnitude of the primary responses (Fig 2A). Our experiments therefore reveal that the availability of an inflamed lymph node during the primary response is important for the generation of an enhanced secondary T-cell response. Because secondary antigen challenge in our experiments involved co-delivery of CpG ODN, causing efficient activation of the draining lymph nodes in both DC-injected and control mice, we deem it unlikely that the already inflamed status of these lymph nodes in DC-injected mice is a key determinant in facilitating the secondary response of the T-cells. The most likely explanation for the stronger secondary response in DC-injected mice is therefore the greater number of CD8+ memory T-cells conserved after contraction of the primary response elicited from the inflamed lymph nodes.

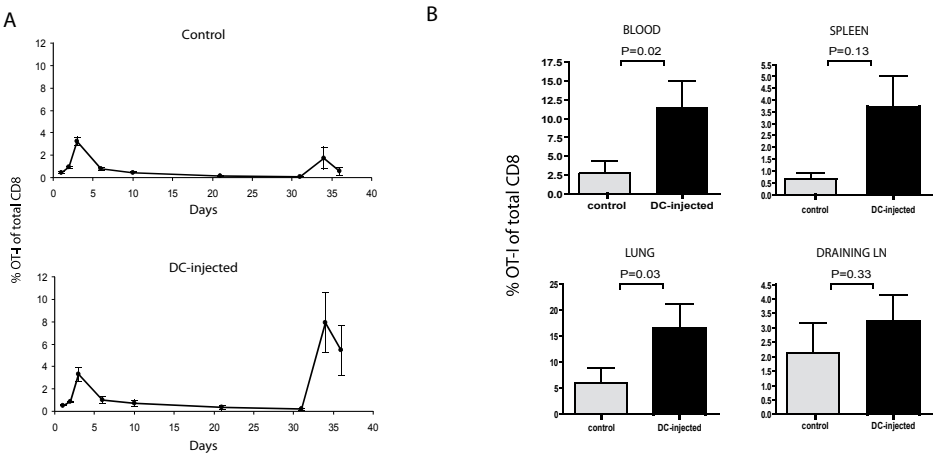

Figure 2: Presence of inflamed LN during primary response increases magnitude secondary T-cell response
Primary responses of ex vivo primed OT-I cells were followed over time in the blood in mice that had received a footpad-injection of LPS-activated DCs, not loaded with antigen, and in control mice. At day 28, after contraction of the primary response, mice were challenged with 50 µg synthetic OVA peptide (SIINFEKL) comprising the OT-I T-cell epitope in combination with 15 µg CpG. This antigen challenge was delivered into the footpad of the right hind leg, at the same site of DC injection. **A.** Kinetics of OT-I in blood in control and DC-injected mice, mean of three mice per group with standard error of the mean. Arrow indicates time of antigen challenge. Representative of three experiments **B.** Mean values for percentages of OT-I cells in different compartments around peak of secondary response (day 8 after secondary challenge) for 14 mice/group. Mann-Whitney test revealed statistically significant differences between groups in blood and lung (p=0.02 and p=0.03 respectively), but not in spleens and draining LNs (p=0.13 and p=0.33 respectively). Two independent experiments performed.

To further analyze the conditions in inflamed lymph nodes in DC-injected mice, we performed footpad injection of CFSE-labeled DCs. As shown in Fig 3A, this results in a rapid increase, within 1 day, of the absolute numbers of various types of cells, including T-cells, B-cells and CD11c+ DCs. The increase in CD11c+ cell numbers cannot be attributed by the injected DCs, because CFSE-high cells were found to constitute less than 0.5% of the CD11c+ population

(data not shown). The host-derived cells recruited to the inflamed lymph node displayed an activated state, as illustrated by the increased frequencies of IL-12 producing CD11c+ cells and TNF-alpha producing CD3+ cells (Fig 3B). There findings strengthened the notion, based on experiments shown in Fig.1C/D, that the injection of activated DCs triggers an overall accumulation of APCs and lymphocytes rather than a selective accumulation and/or activation of antigen-specific OT-I T-cells. Accordingly, we did not find significant differences in the activation status, as determined by IFN-gamma production, of ex vivo primed OT-1 cells between inflamed and resting lymph nodes of DC-injected and control mice respectively, neither in the effector phase, nor in the post contraction phase of the primary response (Fig 3C). Evaluation of additional markers that were found to be associated with T-cell activation and memory cell formation, in particular CD62L, CD127, CD122, CCR7 and TRAIL (17-19), also failed to reveal a qualitative difference between the OT-I T-cells in resting versus inflamed lymph nodes (supplementary figure 1 and data not shown).

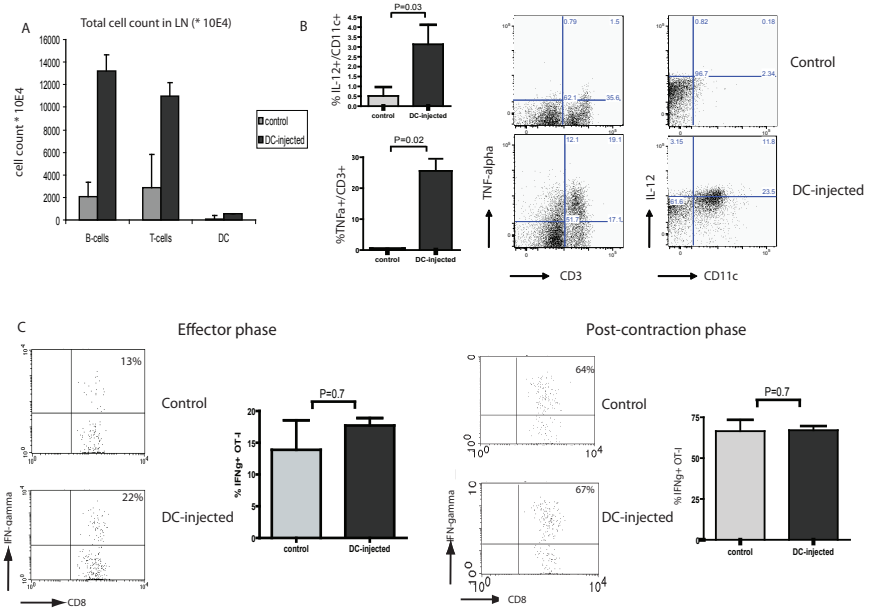

Figure 3: Conditions in the inflamed LN

Cellularity and cytokine production in inflamed and control LN was analyzed 24h after footpad-injection of LPS-activated DC's. Single cell suspensions of LN were stained for CD19, CD3 and CD11c **A**:. Absolute numbers of cells in inflamed and control LN. **B**: Percentage cytokine producing cells in inflamed and control LN 24h after footpad-injection of DC. Cytokine production was analyzed by intracellular cytokine staining following PMA/ionomycin activation. In each experiment 4 mice per group were used. Mann-Whitney test revealed statistically significant differences in IL-12p40 and TNF-alpha production between groups (p=0.03 and p=0.02 respectively). Two independent experiments performed. **C**: IFN-gamma production by OT-I CD8+ T-cells at day 3 and 26 after start of T-cell activation. Single cell suspensions of LN were analyzed by intracellular cytokine staining following 3h stimulation with SIINFEKL peptide in vitro. Mann-Whitney test revealed no significant difference between groups in effector and post-contraction phase (p=0.7 in both phases). Groups of three mice, three independent experiments performed.

The possibility of a qualitative rather than a mere quantitative difference between OT-I cells in DC-injected and control recipient mice, was further investigated by harvesting OT-I memory T-cells from mice with inflamed lymph nodes in the early post-contraction phase (12 days after infusion), and transferring these – in identical numbers - to either DC-injected or control recipients (Fig 4A). T-cells were allowed to rest for another 15 days in new recipients, after which they encountered a secondary antigenic challenge through footpad injection of OVA-peptide/CpG. Analysis of the secondary in vivo OT-I responses showed that the availability of an inflamed lymph node in the recipient mice was of key importance for a strong, systemic memory T-cell response (Fig 4B). Thus, post-contraction OT-I T-cells harvested from inflamed lymph nodes only persisted in greater quantities when transferred into DC-injected recipients, and therefore did not exhibit an intrinsic capacity for survival independent of the availability of an inflamed lymph node. In reciprocal experiments, we found that post-contraction OT-I cells harvested from control mice led to stronger secondary responses in magnitude when infused into DC-injected mice than when infused into control mice (Fig 4C) supporting the notion that inflamed LN supports memory T cell maintenance and CD8+ memory T-cells 'educated' in DC-injected and control mice did not differ intrinsically. In order to determine if the OT-I T cells from DC-injected mice reacted differently to antigenic stimuli, OT-I T-cells cells were recovered, at day 7 and day 24 after adoptive transfer, from lymph nodes of DC-injected and control mice, and co-cultured in vitro with feeders and specific peptides. Proliferation to the specific peptide was analyzed by tritium incorporation. (Fig 4D) Although a trend towards more proliferation at day 7 in the DC-injected group was observed, this difference was not significant. At day 24, no difference between OT-I cells from DC-injected versus control mice to the antigenic challenge was observed. . This supports our hypothesis that the amplified response in vivo in DC-injected mice is due to quantitative difference caused by the larger absolute numbers of antigen-experienced T-cells in the inflamed lymph node.

Importance of memory T-cell conservation by inflamed lymph nodes in anti-viral immunity

So far, our experiments demonstrated that secondary challenge at the site of prior lymph node activation can result in a superior memory T-cell response. In additional experiments, we delivered the secondary antigenic challenge, consisting of ovalbumin peptide and CpG ODN, in the ipsilateral footpad. Antigen delivery at this site resulted in a secondary T-cell response comparable to that elicited by antigen delivery in the foot pad near the inflamed lymph

node, showing that the locally conserved memory T-cells can be recruited into secondary responses triggered by antigenic delivery at distal sites (data not shown). To further assess the physiological significance of memory CD8+ T-cell conservation in inflamed, local lymph nodes, we infected OT-I recipient, DC-injected and control mice intra-tracheally with recombinant influenza virus encoding the ovalbumin antigen (13)

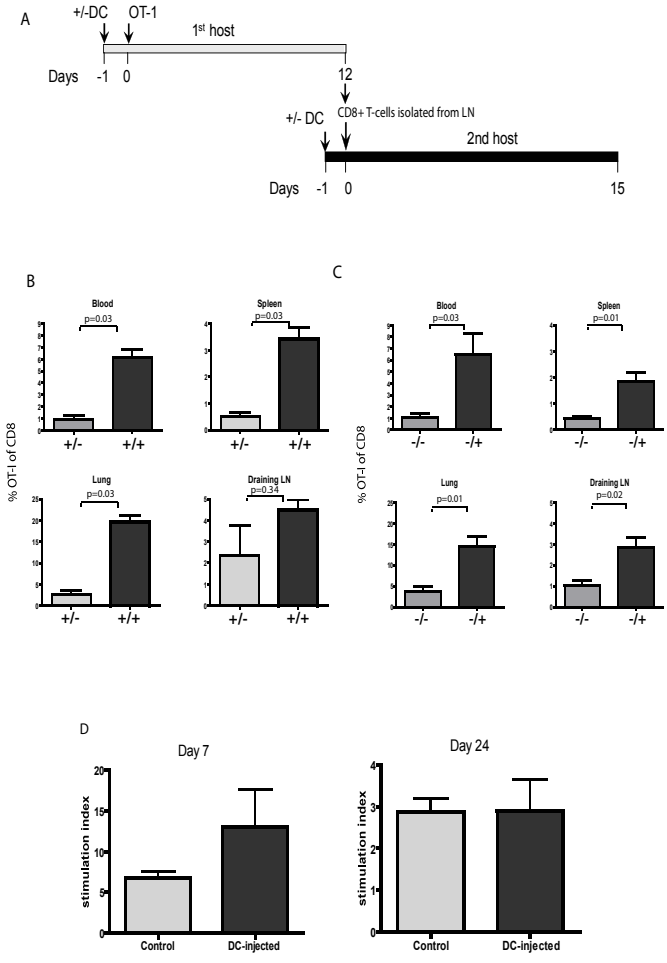

Figure 4: T-cells educated in inflamed LN are not intrinsically changed

T-cells recovered from inflamed or control LN were counted and adoptively transferred in identical numbers (10^3 OT-I cells/recipient) into new recipients with or without inflamed LN. Response to a boost vaccination was analyzed in blood, spleen, lung and draining LN. **A**: Schematic cartoon of experimental design. **B**: Mean values for percentages of OT-I cells educated in mice with inflamed LN in different compartments around peak of secondary response (day 8 after secondary challenge) 4 mice/ group. Mann-Whitney test revealed significant differences for blood, spleen and lung (p=0.03 for all compartments) but not draining LN (p=0.34) between groups. **C**: Mean values for percentages of OT-I cells educated in control mice in different compartments around peak of secondary response (day 8 after secondary challenge) 4 mice/group. Mann-Whitney test revealed significant differences for blood, spleen, lung and draining LN (p=0.03, p+0.01, p=0.01 and p=0.02 respectively) between groups. Two independent experiments performed (A and B)

T-cells recovered from inflamed or control LN at day 7 and day 24 were isolated by flow-cytometric sorting and co-cultured with irradiated spleen cells and SIINFEKL peptide in equal amounts (1250 cells/well for day 7, 500 cells/well for day 24). After 36 hours, cells were pulsed with tritium and incorporation was measured 24 hours later. Stimulation index was calculated as tritium count with peptide divided by tritium count of medium control. **C:** Stimulation index of OT-I cells recovered on day7 and day 24 respectively. Groups of 5 (day 7) and 10 (day 24) mice. Mann-Whitney test revealed no significant differences between groups. Mann-Whitney test revealed no significant differences on day 7 and day 24 (p=0.25 and 1.0 respectively).

As shown in Fig 5, DC-injected OT-I recipients have a significantly lower amount of virus particles in their lungs. This can be explained by the fact that DC-injected recipients develop a memory T-cell response superior to that of control OT-I

recipients, which is capable of more efficiently controlling the viral infection.

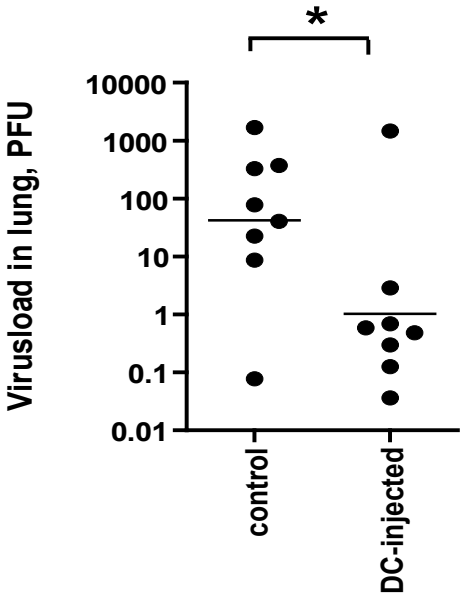

Figure 5: Presence of inflamed LN during primary response enhances anti-viral immunity

A secondary response to an influenza virus containing the SIINFEKL epitope is more vigorous in mice with an inflamed LN, induced by injecting LPS-activated DC not loaded with antigen into the footpad of mice, during the primary response. Mice with an inflamed LN or control mice were adoptively transferred with in vitro activated OT-I T-cells. After 35 days mice were infected with 5×10^4 PFU influenza virus through intra-tracheal injection. 5 days later viral load in the lungs was determined by quantitative PCR. Mann-Whitney test revealed significant reduction of virus RNA in DC-injected mice compared with control mice. (P=0.05) 8 mice per group.

4 Discussion

By separating DC-T-cell engagement and lymph node activation in space and time, we demonstrated that the presence or absence of an activated lymph node has no impact on the magnitude and kinetics of the primary response, at least in the absence of cognate antigen, but clearly influences the magnitude of the memory T-cell response upon secondary antigen encounter. Furthermore, our data show that presence or absence of an activated lymph node does not imprint intrinsic differences into the T-cells that would result in alternate survival or memory programs. Instead, the activated lymph nodes appear to primarily offer increased storage space for antigen-experienced T-cells during the contraction and memory phases of the primary response. This offers opportunities for using lymph node inflammation in conditioning patients for adoptive T-cell transfer strategies. Aswell as the current method of lympho depletion, lymph node inflammation

can create a nurturing environment for newly injected T-cells. Our findings differ from those emerging from studies that have looked at the impact of signals at the DC-T-cell interface on the development of T-cell memory. For instance, the presence of costimulatory signals involving the 4-1BB and OX40 pathways during T-cell priming imprints a survival program that promotes development of T-cell

memory and enhanced secondary T-cell responses(20,21) (22). Furthermore, the presence of CD4+ T-cells during CD8+ T-cell priming was shown to render CD8+ T-cells insensitive to TRAIL-mediated death during the secondary response, thereby endowing them with the capacity to mount an enhanced memory T-cell response (17,23). These prior studies and our present work imply that T-cell programming during DC-T-cell engagement and the availability of enlarged lymph nodes for T-cell storage are expected to synergize in the generation of potent T-cell memory.

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