Title: Transient dendritic cell activation diversifies the T cell response to acute infection

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Abstract: The precise timing of T cell priming during infection remains unclear. Here, we mapped the cellular dynamics of all immune lineages during acute infection with *Listeria monocytogenes* (*Lm*). We identified highly transient DC activation 2 days post-infection that functions as a critical time window for priming effector T cells. Regulation of this transient state was mediated by DC extrinsic IFN γ provided by lymphocytes. Furthermore, antigen-specific T cells that arrive late to the site of priming and miss peak DC activation acquire only memory T cell fates. This temporal regulation of fate is recapitulated by CD8⁺ DCs *ex vivo*, suggesting that shifts in activation state of a single antigen presenting cell population alter T cell fates. These results uncover a novel mechanism for temporal regulation of T cell differentiation during a dynamic immune response to acute infection.

One-Sentence Summary: The immune system generates a balanced protective response through rapidly shifting activation states and recruitment of new cells into an ongoing inflammatory landscape.

One of the most important cellular interactions mediating protective adaptive immune responses is the priming of CD8 T cells by DCs. Upon recognition of cognate antigen presented on Major Histocompatibility Complex class I (MHCI) by DCs, CD8 T cells rapidly divide and differentiate into multiple mature effector populations (1). Early decisions in T cell fate lead to the acquisition of functionally distinct differentiation states including memory and cytotoxic effector phenotypes (1). Intriguingly, single cells are committed to specific fates very early after priming by DCs (2, 3). Because strength of signaling during priming dramatically alters fate acquisition (4), this suggests variability in the strength of signals received by individual T cells during the priming process.

Previous studies have demonstrated that T cells transferred into mice at different timepoints of an immune response undergo different magnitude of expansion and acquire distinct fates (5–7). Adoptive transfer of CD8 T cells at various days post infection (DPI) in vesicular stomatitis (-1, 2, or 4 DPI) and vaccinia (0, 3, or 7 DPI) viral infections demonstrated that T cells primed later in infection proliferate less and acquire a memory fate (5, 6). In chronic lymphocytic choriomeningitis virus (LCMV) infection, CD8 T cells transferred into mice with established chronic infection (21 DPI) divided less and acquired memory fates (7). These data suggest that the signals encountered by T cells fluctuate as a function of time during infection. However, it remains unclear which cellular interactions are responsible for these observations and how they are regulated, though clonal competition has been suggested as a potential explanation.

DCs are highly responsive to environmental stimuli while also possessing several mechanisms to prevent their hyperactivation. Therefore, we wondered whether regulation of DC activation over time might instead contribute to the diversification of T cell fates to balance effector and memory T cell differentiation. We sought to map DC activation throughout infection, identify regulatory mechanisms of DC activation, and define the phenotypic landscape of T cells primed at different stages of DC activation. These experiments yield results with important implications for how DCs induce the differentiation of diverse T cell fates during natural immune responses and how this might be therapeutically manipulated in different contexts.

Immune Landscapes During Acute Infection

To map the complete immune response to acute infection with *Lm* expressing the model antigen ovalbumin (*LmOVA*), wild type C57BL/6 mice were infected intravenously, and spleen tissue was harvested every day for the first 9 days of infection. To quantify the frequencies and activation states of immune cells we used mass cytometry to profile 4,325,404 single cells using a panel of 41 antibodies designed to identify all mature immune lineages with the addition of several markers that specify activated states of antigen presenting cells and T cells (Fig. S 1) (8, 9). We identified distinct cell states through unsupervised clustering and observed highly transient immune landscapes throughout different stages of infection (Fig. 1A-D, Fig. S 2A). We saw modest compositional changes 1 day post infection (DPI) followed by more substantial innate immune activation from 2-5 DPI and the emergence of activated T cells from 6-9 DPI (Fig. 1A-D). We found equivalent immune landscapes when assessing compositional changes based on manually gated population frequencies and unsupervised cluster frequencies (Fig. S 2B-C).

Quantifying compositional changes by Aitchison distance, we found that the global composition of immune cells remained highly distinct from uninfected animals throughout infection, and

substantial changes were evident between each subsequent day, underscoring the dynamicity of the response (Fig. 1E). Compositional changes between consecutive days began to decrease at 6 DPI with the smallest changes seen between 8-9 DPI (Fig. 1E). Activated CD8 T cells, a critical adaptive immune cell type required for clearance of *Lm* infection (10, 11), began to emerge in significant frequencies at 5 DPI followed by the acquisition of terminally differentiated fates (Fig. 1F, Fig. S 2D). This suggests that the key T cell priming event occurs early during infection.

Dendritic Cell Activation During Acute Infection

Given the dynamic changes to innate immune populations early in infection, we next sought to assess the activation dynamics of type 1 conventional dendritic cells (cDC1s) during *Lm* infection, the principal cell type that primes CD8 T cells in *Lm* (12). Consistent with previous findings, we found that *Batf3*^{-/-} mice lacking cDC1s exhibited deficient CD8 T cell responses to *Lm* infection (Fig. 2A). To further assess the activation state of cDC1s over time, we calculated a composite activation score based on the expression of key molecules associated with activation. We found that cDC1 activation reached a transient peak at 2 DPI that was significantly higher than the previous or subsequent day (Fig. 2B-C). Variance in cDC1 activation score was also highest at 2 DPI suggesting cDC1s from this timepoint comprise the widest range of possible activated states that an individual T cell could interact with (Fig. 2B). We also found that cDC1s presenting SIINFEKL:H2-K^b complexes (an immunodominant OVA antigen in the context of MHC class I) on the cell surface were most abundant at 2 DPI as measured by flow cytometry (Fig. 2D, Fig. S 3A). The limit of detection for 25-D1.16 SIINFEKL:H2-K^b staining is greater than that for CD8 T cell activation (13), thus these data indicates that cDC1s at 2 DPI have the highest levels of cell surface SIINFEKL:H2-K^b but does not suggest that SIINFEKL:H2-K^b complexes are absent at other timepoints. We further validated transient cDC1 activation in mouse cytomegalovirus (MCMV) infection and found that cDC1s were similarly most activated at 2 DPI (Fig. 2E).

To map cDC1 activation more comprehensively, we utilized mass cytometry with an extended panel of activation molecules and used PHATE dimensionality reduction (14) along with Slingshot lineage reconstruction (15) (Fig. 2F-H). We calculated a Pseudotime activation trajectory, which was characterized by increasing expression of all activation markers from minimum to maximum Pseudotime (Fig. 2 F-H). Activation and costimulatory molecules had distinct patterns of upregulation; for instance, CD86 expression steadily increased throughout the trajectory while CD70 expression only increased in cells at the maximum end of the Pseudotime trajectory (Fig. 2 G-H). We found similar gradients of cDC1 activation from MCMV infected mice (Fig. S 2B-C). Thus, a unique cDC1 activation state transiently arises only at 2 DPI, which represents the most highly activated cDC1 state during acute infection.

Cell Extrinsic Regulation of Transient Dendritic Cell Activation

To identify the mechanisms by which peak cDC1 activation is regulated at 2 DPI, we first quantified cytokines from spleen tissue homogenate during early infection. We found that samples from 2 and 3 DPI had markedly different cytokine landscapes from each other and from uninfected and 1 DPI samples (Fig. 3A, Fig. S 4A). Specifically, IFN γ levels peaked at 2 DPI and were the primary cytokine driving differences in the cytokine landscape (Fig. 3B). A previous study found that IFN γ signaling mediates IL-12 production by DCs (16), supporting an important role for this pathway. The STAT1 signaling pathway is prototypically activated by IFN γ (17). Therefore, we quantified levels of phosphorylated STAT1 (pSTAT1) at 2 DPI in

cDC1s and found that cDC1s had elevated levels of pSTAT1, suggesting that IFN γ signaling was acting directly on cDC1s (Fig. 3C).

To assess whether cell-intrinsic recognition of *Lm* by pattern recognition receptors activated STAT1 signaling we incubated cDC1s with heat killed *Lm* (HKLM) and identified signaling responses in I κ B α , pERK, and pP38 (Fig. S 4B). However, we found no change in pSTAT1, demonstrating that cDC1s pSTAT1 signaling at 2 DPI is via cell-extrinsic IFN γ (Fig. 3C). To validate our findings of transient IFN γ levels and to identify the cellular source of IFN γ , we utilized an IFN γ GFP reporter mouse (18) and found that splenic GFP⁺ IFN γ producing cells are undetectable at 1 DPI but prominent at 2 DPI (Fig. 3D, Fig. S 4C). We further found that the majority of IFN γ producing cells at 2 DPI were T and NK cells (Fig. 3E, Fig. S 4D). We then depleted NK cells or Thy1⁺ lymphocytes and observed that only Thy1 depletion significantly decreased IFN γ levels in the spleen at 2 DPI (Fig. 3F, Fig. S 4E-F). Depletion of Thy1⁺ cells and IFN γ both significantly decreased splenic cDC1 activation at 2 DPI (Fig. 3 G-H). Together these data suggest that peak cDC1 activation is dependent on cell extrinsic IFN γ produced by lymphocytes.

Transient Dendritic Cell Activation Regulates T Cell Fate

Next, we sought to understand the functional consequences of transient cDC1 activation. We reasoned that all T cells capable of recognizing a specific antigen during acute infection are dispersed throughout the organism and that priming occurs in specific secondary lymphoid organs (SLO) where antigens are present. Using relevant parameters from the literature, we developed a computational model of T cells encountering SLOs as a function of time post-infection utilizing a multinomial sampling procedure (Methods). Our simulations showed that, throughout an acute infection, naïve antigen specific T cells continually enter priming SLOs over time (Fig. 4A), even after accounting for inflammation induced recirculation which increases the rate of T cell trafficking (19, 20). Thus, a meaningful fraction of naïve T cells will likely arrive late to the site of priming, after 2 DPI (Fig. 4A). Based on our experimental observations, these late arriving antigen specific cells will encounter a dramatically shifting landscape of antigen presenting cells. Consistent with this result, we found that inhibiting lymphocyte trafficking using FTY720 reduced the number of splenic CD8 T cells that exhibited evidence of recent TCR signaling, using a reporter for Nur77, on 3 DPI (Fig. S 5A).

To experimentally test the effects of transient cDC1 activation, we adoptively transferred TCR transgenic OT1 CD8 T cells at 0-4 DPI with *Lm*-OVA and then assessed OT1 fate in the spleen 7 days after transfer (Fig. 4B). In this model system, cells transferred in at 3 and 4 DPI miss the transient cDC1 activation peak on 2 DPI. We found that the magnitude of T cell expansion was significantly decreased over time, as measured by the total numbers of splenic OT1 CD8 T cells (Fig. 4D). We also found that OT1 cell fate shifted from primarily KLRG1⁺CD62L⁻ short-lived effector cell (SLEC) fate to KLRG1⁺CD62L⁺ central memory fate as a function of time (Fig. 4C,E,F), and SLEC differentiation was entirely lost after 2 DPI. We delved deeper into the phenotypes of 0 and 3 DPI CD8 T cells using an extended antibody panel and found that cells transferred at each timepoint differentiated into unique cell states (Fig. 4G, Fig. S 5B). The memory phenotype exhibited by 3 DPI cells was uniquely characterized by expression of CD90, CD127, TCF1 and CD62L (Fig. S 5B). The effector phenotype exhibited by 0 DPI cells was uniquely characterized by expression of PD1, Ki67, CD25, GrzmB, KLRG1, and CD11c (Fig. S 5B). We found identical results utilizing low affinity OT3 CD8 TCR transgenic T cells (21) with *Lm*-OVA infection as well as with adoptive transfer of polyclonal T cells in the context of MCMV infection (Fig. 4H-K, Fig. S 5C-D). This suggests that transient DC activation regulates

the balance between SLEC and memory T cell differentiation regardless of TCR affinity and across infection contexts. We confirmed that OT1 T cells primed at 3 DPI formed functional memory by assessing the capacity for memory T cells to produce multiple effector cytokines as well as the capacity to expand upon rechallenge in new hosts (Fig. 4L-M).

To assess how early the memory fate is imprinted on 3 DPI transferred OT1 T cells, we sorted T cells 72 hours after adoptive transfer and performed RNA sequencing. We found that T cells were already proliferating at this time point (Fig. S 5E), and T cells transferred at 0 and 3 DPI were already transcriptionally distinct from one another and from naïve CD8 T cells (Fig. 4N-O). We identified 2,403 genes upregulated in 3 DPI transferred cells and 1,792 upregulated genes in 0 DPI transferred cells (Fig. S 5F). Specifically, genes known to be associated with memory cell fate, such as *Tcf7* and *Ccr7*, were upregulated in cells transferred 3 DPI, whereas genes associated with cytotoxic effector fate, such as *Ifng* and *Gzmb*, were upregulated in cells transferred 0 DPI (Fig. 4O). Geneset Enrichment Analysis further identified that cells transferred 0 DPI exhibited a highly proliferative state characterized by higher expression of genes in ribosome biogenesis, RNA transport, oxidative phosphorylation, and carbon metabolism (Fig. S 5G).

While cDC1 activation dynamics coincide precisely with this change in CD8 T cell fate, it remained possible that other cell types were contributing to this regulation. To assess whether changes in cDC1 activation state alone were sufficient to drive alternative CD8 T cell fates, we isolated cDC1s from 1.5 or 2.5 DPI and cocultured them *ex vivo* with naïve OT1 T cells. We found that CD8 T cells that were primed by cDC1s from 2.5 DPI were significantly more likely to retain CD62L expression (Fig. 4P-Q). These results indicate that changes in cDC1 activation state within this narrow but critical window of time are sufficient to alter CD8 T cell fate.

Summary and Conclusions

To understand how innate immune cells orchestrate an adaptive immune response, we employed high dimensional mass cytometry to generate a comprehensive map of the splenic immune response to infection. Unsupervised analysis of these data allowed for the identification of highly transient cell states across lineages. These data revealed a remarkably transient activation state for cDC1s at 2 DPI with *Lm* and MCMV, characterized by peak expression of costimulatory molecules as well as MHC peptide complexes. By quantifying intrasplenic cytokines, we found that peak cDC1 activation is supported by lymphocyte-derived IFN γ . Finally, we probed the functional consequences of transient cDC1 activation. We found that T cells primed at different stages of cDC1 activation expand to different population sizes and acquire different fates. Specifically, T cells primed after peak cDC1 activation exclusively acquire memory fates. Taken together, these data suggest that transient cDC1 activation in acute infection acts as a temporal regulator of CD8 T cell fate and contributes to the diversity of T cell fates that ensure both a productive primary immune response as well as the development of immunological memory.

While previous studies have observed changes in T cell fates based on the timing of adoptive transfer, the underlying mechanisms remained enigmatic due to a lack of context of the immune response dynamics across cell types. Here, we show that transient DC activation is a critical mechanism that drives diversification of CD8 T cell fates and is sufficient to explain this previously mysterious observation. Importantly, we designed our time course of T cell transfers based on the transient DC activation state we identified and utilized high dimensional technologies to deeply profile T cells primed at different phases of DC activation. In addition, we confirmed that changes in the cDC1 activation state are sufficient to drive different T cell

differentiation trajectories using an *in vitro* reductionist system. Our work extends and contextualizes previous findings by comprehensively mapping the activation states of cDC1s during acute infection over time, as well as the range of activation within each timepoint. Bystander T cells and NK cells produce IFN γ early in acute infection to support host defense (22, 23). Here, we identified a new role for lymphocyte-derived IFN γ early in regulating the kinetics of cDC1 activation to support the ultimate expansion of antigen specific T cells and to regulate their balance in fates.

Elegant studies tracing the fates of single T cells have shown striking differences in expansion and fate of individual expanded clones, even when cells are transferred at different times during infection (24, 25). Since T cell fate is imprinted within the first cell division (2, 3), our study shows that the range of DC activation states throughout infection produce diverse T cell fates. Even for T cells that encounter cDC1s at the peak of their activation (2 DPI), there is a large range of highly to lowly activated cDC1s that a T cell might encounter at this time, which may thus imprint different fates. Each DC will thus express a unique combination of costimulatory molecules at varying levels as well as varying levels of MHC-peptide complexes. Future studies should build on this paradigm and trace the fates of individual T cells as a function of the activation state of their priming DCs as well as dissect how different costimulatory molecule landscapes instruct T cell differentiation.

We envision this study as a conceptual framework for studying immune responses as rapid time courses of high-dimensional single-cell data that can reveal novel mechanistic insights for how the immune system makes decisions. Our findings suggest that the structure of the immune system is a key mechanism for providing balanced protection to the host. A widely dispersed network of immune cells across tissues that undergoes waves of activation and migration utilizes time and space to provide a diverse T cell response that can both eradicate disease quickly and provide long term protection. Understanding the transient activation of DCs has broad consequences for therapeutic interventions, and we envision this work will inform future therapeutic strategies to harness these properties to tune T cell responses in infection, cancer, or autoimmunity.

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Data and materials availability: Mass cytometry and RNA-seq data will be made publicly available through FlowRepository and GEO. All relevant code for figures in the paper will be available upon request.

Supplementary Materials

Materials and Methods

Figs. S1 to S5

Tables S1 to S2

Fig. 1: Immune landscape shifts during acute infection. (A) Principal component analysis of mouse spleen population frequencies throughout infection measured by mass cytometry. Populations were identified by unsupervised clustering. **(B)** Normalized frequencies as a percent of total cells for all unsupervised clusters in each mouse. **(C)** UMAP dimensionality reduction of all cells measured across samples colored by major landmark population. **(D)** Cells from each timepoint superimposed on the total immune landscape. **(E)** Quantification of compositional changes throughout infection by Aitchison distance. Splenic landscapes from each timepoint are compared to either uninfected mice or the preceding timepoint. **(F)** Total frequencies of splenic CD8 T cell subsets identified by unsupervised clustering.

Fig. 2: cDC1 activation states during acute infection. (A) Representative scatter plots and quantification of splenic cytotoxic effector CD8 T cells at 7 DPI with *Lm*-OVA in wild type or BATF3^{-/-} mice. (B) Composite activation score (CD80, CD86, CD83, CD54, CD69, PDCA1, CD62L, and PDL1) in single cDC1s plotted throughout infection with *Lm*-OVA. Variance at each timepoint quantified in italics. (C) Normalized expression of all molecules used to calculate activation score. (D) Percent of cDC1s expressing high levels of MHCII-SIINFEKL complexes during early infection with *Lm*-OVA. (E) Composite activation score (CD80, CD86, CD83, CD54, CD69, PDCA1, CD62L, H2-K^b, CD70, MHC II and PDL1) in single cDC1s plotted throughout infection with MCMV. Variance at each timepoint quantified in italics. (F) PHATE dimensionality reduction of cDC1s at 2 DPI with *Lm*-OVA colored by Pseudotime. (G) Expression of key costimulatory and activation markers in cDC1s. (H) Normalized protein expression grouped into 20 bins based on pseudotime. Fold change in expression from minimum to maximum Pseudotime plotted on the x-axis and number of cells in each Pseudotime bin plotted on the y-axis.

Fig. 3: T cell derived IFN γ supports peak cDC1 activation. (A) Principal component analysis of mouse spleen cytokines during early *Lm*-OVA infection. (B) Vector plot of top 15 cytokines contributing to PC1 and PC2 colored by contribution. (C) pSTAT1 expression from splenic cDC1s at 2 DPI with *Lm*-OVA or uninfected mice and after 15 minutes of incubation with HKLM *ex vivo* or unstimulated. (D) Quantification of GFP $^{+}$ cells in the spleen of IFN γ -GFP reporter mice after infection with *Lm*-OVA. (E) Quantification of the cellular identity of GFP $^{+}$ cells at 2 DPI with *Lm*-OVA. (F) Quantification of splenic IFN γ at 2 DPI with *Lm*-OVA after antibody depletion of NK cells or Thy1 $^{+}$ lymphocytes. (G) Normalized mean expression of key proteins in splenic cDC1s at 2 DPI with *Lm*-OVA following depletion of T cells or IFN γ . (H) Composite activation score (CD80, CD86, CD83, CD54, CD69, PDCA1, CD62L, H2-K b , CD70, MHC II and PDL1) in splenic cDC1s at 2 DPI with *Lm*-OVA following depletion of lymphocytes or IFN γ .

Fig. 4: Late-arriving CD8 T cells acquire memory fates. (A) Simulation depicting the cumulative proportion of antigen specific T cells that pass through a SLO capable of priming. (B) Schematic for experimental testing of late arriving T cell differentiation capacities in which OT1 CD8 T cells are adoptively transferred into mice 0-4 DPI with *Lm*-OVA and then harvested after 7 days. (C) Representative scatter plots of CD8 T cells after 7 days of differentiation from experiment depicted in (B). (D) Quantification of splenic OT1 CD8 T cell expansion by DPI of adoptive transfer in *Lm*-OVA. Quantification of splenic KLRG1⁺ SLEC (E) or CD62L⁺ central

memory cells **(F)** OT1 CD8 T cells by DPI of adoptive transfer in *Lm-OVA*. **(G)** UMAP dimensionality reduction of equal numbers of OT1 CD8 T cells transferred at 0 or 3 DPI and then harvested from spleens 7 days after transfer. Quantification of splenic KLRG1+ SLEC **(H)** or CD62L+ central memory **(I)** OT3 CD8 T cells by DPI of adoptive transfer in *Lm-OVA*.
5 Quantification of splenic KLRG1+ SLEC **(J)** or splenic CD62L+ central memory **(K)** polyclonal CD8 T cells by DPI of adoptive transfer in MCMV. **(L)** Representative histograms of 0 or 3 DPI *Lm-OVA* transferred memory OT1 CD8 T cells restimulated with PMA & Ionomycin 40 DPI. **(M)** Quantification of 0 or 3 DPI *Lm-OVA* memory OT1 T cells retransferred into new hosts at 40 DPI and rechallenged. Cell counts are 7 DPI rechallenge. **(N)** Principal component analysis of
10 RNA-seq of naïve and 0 or 3 DPI *Lm-OVA* primed OT1 T cells 72 hours after transfer. **(O)** Normalized expression of differentially expressed genes known to be involved in memory or effector fates. Quantification of CD62L⁻ effector **(P)** or CD62L⁺ memory **(Q)** OT1 T cells primed *ex vivo* by cDC1s from 1.5 or 2.5 DPI *Lm-OVA*.