Differentiation of distinct long-lived memory CD4 T cells in intestinal tissues after oral *Listeria monocytogenes* infection

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Mucosal antigen-specific CD4 T-cell responses to intestinal pathogens remain incompletely understood. Here we examined the CD4 T-cell response after oral infection with an internalin A 'murinized' *Listeria monocytogenes* (*Lm*). Oral *Lm* infection induced a robust endogenous listeriolysin O (LLO)-specific CD4 T-cell response with distinct phenotypic and functional characteristics in the intestine. Circulating LLO-specific CD4 T cells transiently expressed the 'gut-homing' integrin $\alpha_4\beta_7$ and accumulated in the intestinal lamina propria and epithelium where they were maintained independent of interleukin (IL)-15. The majority of intestinal LLO-specific CD4 T cells were CD27$^-$ Ly6C$^-$ and CD69$^+$ CD103$^-$ while the lymphoid LLO-specific CD4 T cells were heterogeneous based on CD27 and Ly6C expression and predominately CD69$^-$. LLO-specific effector CD4 T cells transitioned into a long-lived memory population that phenotypically resembled their parent effectors and displayed hallmarks of residency. In addition, intestinal effector and memory CD4 T cells showed a predominant polyfunctional Th1 profile producing IFNγ, TNFα, and IL-2 at high levels with minimal but detectable levels of IL-17A. Depletion of CD4 T cells in immunized mice led to elevated bacterial burden after challenge infection highlighting a critical role for memory CD4 T cells in controlling intestinal intracellular pathogens.

INTRODUCTION

The intestinal mucosa is one of the largest surfaces of the body. One important function of the intestinal mucosa is to provide a niche for commensal bacteria, which helps to metabolize ingested food and shapes immune responses. Lymphocytes that participate in intestinal immune responses are usually induced in gut-associated lymphoid tissues (GALT), such as the Peyer's patches (PP), or the intestinal draining mesenteric lymph nodes (MLN) in order to act at effector sites such as the lamina propria (LP) or within the intestinal epithelial cell layer as intraepithelial lymphocytes (IEL).[1] CD4 T cells are one of the major immune cell subsets located within these distinct compartments where they perform a wide range of functions. Substantial knowledge of intestinal CD4 T-cell responses has been established in regard to inflammatory disorders and commensals. However, to better understand the nature of protective immunity to enteric pathogens it is critical to define the functional profile of intestinal pathogen-specific

effector and memory CD4 T cells responding to an oral infection.

Depending on environmental signals, naive CD4 T cells can differentiate into effector subsets with unique functional profiles classified by preferential production of key cytokines into T-helper (Th) subsets. Th1 cells provide protection against intracellular pathogens primarily through secretion of IFNγ. On the other hand, Th2 cells provide protection against extracellular pathogens through secretion of interleukin (IL)-4, -5, and -13, which promotes humoral responses. The diversity of CD4 T-cell subsets increased after the identification of regulatory T cells, Th17, Tfh, Th22, and Th9.[2] Regulatory T cellss regulate immune responses through multiple mechanisms including secretion of IL-10. Th17 cells secrete IL-17 family cytokines to regulate immunity to extracellular bacteria.[3] Initial *in vitro* studies into Th differentiation suggested that these lineages were distinct with little or no plasticity after polarization. However, recent evidence in vivo has suggested

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that Th-cell differentiation and lineage commitment is more dynamic than previously appreciated.[4] In fact, functionally dynamic phenotypes of circulating CD4 T cells were confirmed by following antigen-specific human memory CD4 T-cell responding to pathogens or vaccination.[5] In addition, route of infection may dictate the differentiation of Th subsets *in vivo* as intravenous (i.v.) *Lm* infection primarily induced Th1 cells, whereas intranasal infection induced Th17 cells.[6] Thus, it is important to define which subsets of CD4 T cells are involved in protection against intestinal bacterial infections and what impact route of infection has on Th lineage decisions.

CD4 T-cell memory was first characterized by markers found on circulating human T-cell populations.[7] This finding, later confirmed in animal models,[8] coined the idea of central memory T cells (Tcm) defined by the expression of lymph node homing markers CD62L and CCR7. Memory CD4 T-cell lacking these markers were termed effector memory T cells (Tem) and are largely found to recirculate through blood and peripheral tissues. Memory CD4 T cells can also reside in non-lymphoid tissues[9] and are thought to be maintained independent of recirculation. Owing to the complexity of CD4 heterogeneity and the difficulty in tracking endogenous pathogen-specific CD4 T cells, it has remained difficult to define markers which identify effector CD4 T cells that are destined to become memory T cells. In addition, the majority of studies have focused on examining lymphoid or circulating cells despite the notion that many protective lymphocytes are located outside these compartments.[10–12] Markers such as CD27, Ly6C and PSGL-1 have been used in various infection settings to define subsets of CD4 T cells with memory potential in lymphoid tissues.[6,13–15] In particular, CD69 and KLRG-1 have been used to identify different subsets of resident memory CD4 T cells in the lung.[11,16] As such, these markers provide a useful tool to examine the phenotype and function of mucosal CD4 T-cell populations responding to infection in the intestinal mucosa.

In this study, we investigated the features of endogenous pathogen-specific CD4 T cells after oral infection with a 'murinized' *Listeria monocytogenes* (*Lm*) to define the Th profile and characterize CD4 T-cell memory formation in the intestinal mucosa. We performed oral infection using a recombinant *Lm* (*rLm*) in which modified internalin A (InlA[M]) protein promotes intestinal invasion of murine epithelial cells thereby recapitulating the route of human infection.[17] This model of oral *Lm* infection has provided great insight into *Lm* pathogenesis and immune responses in the intestinal mucosa.[18,19] In C57Bl/6 (B6) mice, naive CD4 T cells can respond to the immunodominant I-A[b]-restricted lister-iolysin O 190-201 (LLO) epitope providing a platform to evaluate endogenous T-cell responses within the intestinal mucosa. After oral InlA[M] r*Lm* infection LLO-specific CD4 T cells migrated into the intestinal mucosa where they adopted a distinct intestinal phenotype. Splenic LLO-specific CD4 T-cell phenotype was highly heterogeneous while a much more homogenous population was established in the intestinal tissues. Once established, intestinal LLO-specific CD4 T-cell

population remained remarkably phenotypically stable throughout all phases of the immune response while maintaining a striking difference to lymphoid LLO-specific CD4 T cells. The cytokine profile was characterized predominantly by the presence of polyfunctional LLO-specific CD4 T cells in all tissues. Most importantly, memory CD4 T cells significantly contributed to protection from a secondary infection highlighting their importance in the intestinal mucosa. These results are particularly relevant as they establish significant benchmarks in the evaluation of intestinal memory CD4 T-cell populations and identify the phenotype and functionality of pathogen-specific CD4 T-cell subsets capable of providing critical protection against an intestinal human pathogen.

RESULTS

Induction of robust LLO-specific mucosal memory CD4 T cells

Previous studies utilizing *Lm* infection models have identified substantial differences between T-cell populations generated from distinct routes of infection with the identical pathogen.[6,18,19] In addition, we have demonstrated the impact of the intestinal environment to influence T-cell lineage decisions.[19] Given the striking differences observed in these studies, we sought to examine the impact of the intestinal environment on CD4 memory development using a novel feeding model of a mouse-adapted *Lm* pathogen. B6 mice were fed InlA[M] r*Lm* to mimic human consumption of contaminated food and limit non-intestinal exposure of Listeria to the blood stream that may occur after gavage.[20,21] The internalin A mutation allows *Lm* to bind murine E-cadherin on intestinal epithelial cells with high affinity recapitulating the route of listeria invasion in humans.[17] In this system, InlA[M] r*Lm* is cleared by ∼7 days after primary infection and 5 days after challenge infection of immunized mice providing a robust system to evaluate T-cell memory (data not shown). MHC class II tetramers were used to identify endogenous *Lm*-elicited LLO-specific CD4 T cells after oral InlA[M] r*Lm* infection. LLO-specific CD4 T cells were detected in the blood as early as 6 days post infection (d.p.i.) and peaked at ∼9 d.p.i. (**Figure 1a,b**). Expression of the gut homing integrin $\alpha_4\beta_7$ mediates T- and B-cell migration into the small intestine through interaction with its ligand, MAdCAM-1.[22] The expression of $\alpha_4\beta_7$ on circulating LLO-specific CD4 T cells peaked with the earliest detection of LLO-specific CD4 T cells at 6 d.p.i. and decreased steadily until 9 d.p.i. (**Figure 1c**). Consistent with this window of $\alpha_4\beta_7$ expression, a substantial population of LLO-specific CD4 T cells was readily identified in the intestinal tissues at 9 d.p.i. (**Figure 1d,e**). Even at steady state, the LP has a preponderance of CD4 T-cell subsets. However, the vast majority of T cells in the IEL compartment express CD8α and not CD4 in naive mice.[23] After oral InlA[M] r*Lm* infection, a large population of LLO-specific CD4 T cells accumulated in the IEL compartment (**Figure 1d–h** and **Supplementary Figure S1** online). Since a robust pathogen-specific effector CD4 T-cell response accumulated in the intestinal compartment, we examined the maintenance and recall capacity of LLO-specific memory CD4 T cells in intestinal
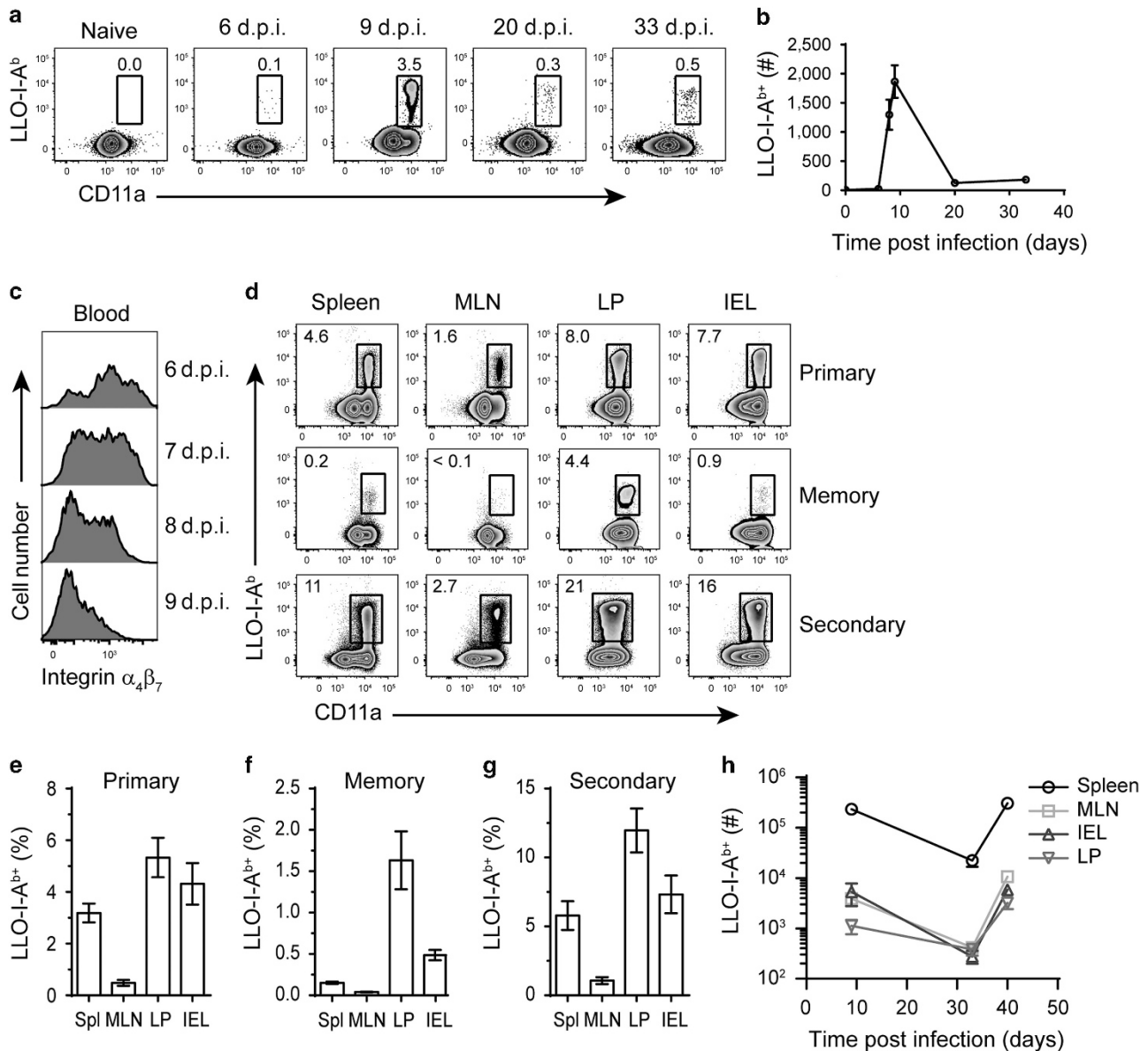
Figure 1 Induction of robust listeriolysin O (LLO)-specific mucosal CD4 T cells. (**a–c**) C57Bl/6 (B6) mice were orally infected with 2×10^9 colony-forming units (CFU) InlA[M] r*Lm* and LLO-specific CD4 T cells were quantified and examined for $\alpha_4\beta_7$ expression from the blood at the indicated times. Representative zebra plots and histograms are gated on live CD4 T cells (**a**) or live LLO-I-A[b +] CD4 T cells (**c**). Absolute numbers of circulating LLO-specific CD4 T cells were calculated per 1 ml of blood (**b**). Data are representative of three experiments with at least three mice per group. (**d–h**) B6 mice were orally infected with InlA[M] r*Lm* and LLO-specific CD4 T cells were quantified from the spleen, mesenteric lymph nodes (MLN), lamina propria (LP), and intraepithelial lymphocyte (IEL) compartment after infection. (**d**) Representative zebra plots are gated on live CD4 T cells at 9 (primary) and 60 (memory) days post infection (d.p.i.), or 7 days after a secondary challenge of mice that were immunized 60 days previously (secondary). Numbers within quadrants correspond to the percentage of cells within gates. (**e**) The graph shows pooled data from five independent experiments with 15–17 mice total at 9 d.p.i. (primary). (**f**) The graph shows pooled data from three independent experiments with 12 mice total between 50 and 90 d.p.i. (memory). (**g**) The graphs showed pooled data from three independent experiments with 8–9 mice total at 7 days after secondary infection of mice immunized 60 days previously (secondary). (**h**) Absolute numbers of LLO-specific CD4 T cells were quantified in the spleen, MLN, LP, and IEL with tetramer enrichment and counting beads at the indicated time post infection. Mice were given a secondary challenge infection with oral InlA[M] r*Lm* at 33 d.p.i. Data are representative of three independent experiments. All graphs depict the mean ± s.e.m.

tissues after oral InlA[M] r*LM* infection. Memory CD4 T cells were generated and maintained in all tissues examined (**Figure 1d,f**). Upon secondary infection, LLO-specific memory CD4 T cells generated a rapid and robust secondary response to challenge infection (**Figure 1d,g,h**). Thus, LLO-specific CD4 T cells were generated after oral InlA[M] r*Lm*

infection that migrated to the intestinal LP and IEL compartments, were maintained and rapidly expanded upon pathogen reexposure. As CD4 T cells have been reported to upregulate the CD8α correceptor when migrating into the IEL compartment and develop into CTL,[24,25] we asked whether different subsets of LLO-specific CD4 T cells within the IEL compartment could

be identified on the basis of CD8α or granzyme B expression after oral infection. However, in this system the overwhelming majority of LLO-specific CD4 IELs did not express CD8αα or CD8αβ (**Supplementary Figure S2a,b**) suggesting that pathogen elicited CD4 T-cell subsets may be distinct from diet-elicited CD4 T-cell subsets.[24] In addition, levels of granzyme B expression on LLO-specific CD4 IELs were not different from its splenic expression (**Supplementary Figure S2c**). These data demonstrate the presence of a distinct pathogen-elicited mucosally focused CD4 T-cell response to oral *Lm* infection and highlight their rapid recall response to a subsequent challenge infection.

Intestinal pathogen-specific CD4 T cells are distinct from their splenic counterparts

The profound influence of tissue location on some cellular phenotypes is well documented. Therefore, we examined the phenotype of intestinal mucosa and splenic CD4 T cells after oral InlA^M rLm infection in wild-type (WT) mice. CD27, Ly6C and KLRG1 have been utilized to identify cellular fate of Th subsets in various models.[6,14–16] Therefore, their surface expression was examined in intestinal and splenic compartments after oral InlA^M rLm infection to determine whether these markers can be used to distinguish different subsets of mucosal effector and memory CD4 T cells. At the peak of the T-cell response (9 d.p.i.), LLO-specific CD4 T cells in the spleen and MLN were heterogeneous with regard to Ly6C and CD27 expression. In stark contrast, the overwhelming majority of intestine infiltrating LLO-specific CD4 T cells did not express Ly6C or CD27 (**Figure 2a–c**). Only small subsets of Ly6C^+ cells transiently appeared in the LP and IEL following bacteria challenge. One potential explanation for these observations is the preferential migration of CD27^− Ly6C^− cells into the intestines. Indeed, a higher percentage of circulating LLO-specific CD4 T cells expressed integrin α4β7 early after oral InlA^M rLm infection when a lower percent of CD4 T cells expressed CD27 and Ly6C (**Figure 2c**). Despite this observation, α4β7 expressing LLO-specific CD4 T cells contain similar proportions of Ly6C and CD27 subsets as α4β7^− LLO-specific CD4 T cells (**Figure 2d**) suggesting that the phenotype of LLO-specific CD4 T cells is altered after migration into the intestinal tissues. Interestingly, LLO-specific CD4 T cells maintained their tissue-specific phenotypes during progression to memory regardless of their tissue location (**Figure 2a–c**). Even after secondary challenge, responding LLO-specific CD4 T cells predominately maintained their phenotypes with subtle but significant alterations in CD27 and Ly6C expression observed (**Figure 2a–c**). A small subset of effector cells in lymphoid tissues and the LP expressed KLRG1. However, this population was not maintained in intestinal tissues and was also reduced in the lymphoid compartment during memory homeostasis (**Supplementary Figure S3a**). Therefore, examination of LLO-specific CD4 T cells responding to oral InlA^M rLM infection identified an unappreciated distinction between circulating and intestinal CD4 populations with regard to KLRG1, CD27, and Ly6C expression.

Several receptors expressed on the surface of T cells are thought to be important for the migration or retention of T cells in peripheral non-lymphoid tissues.[1] CD103 is thought to be involved in CD8 T-cell retention in epithelial layers through interactions with its ligand E-cadherin.[26–28] However, epithelial derived CD4 T cells do not typically express CD103 and several studies have demonstrated CD103-independent retention of resident memory T (Trm) cells.[11,19,29,30] CD69 has also been implicated in the regulation of T-cell migration and retention in peripheral tissues.[10,27,31] In addition, P-selectin glycoprotein ligand-1 (PSGL-1) is critical for the recruitment of Th1 CD4 T cells to the intestinal mucosa.[32] To gain insights into the role of these pathways on the migration or retention of pathogen-specific CD4 T cells in intestinal tissues, mucosal effector and memory LLO-specific CD4 T cells were examined for expression of CD103, CD69, and PSGL-1. First, most LLO-specific CD4 T cells expressed high levels of PSGL-1 in all tissues after oral InlA^M rLm infection and PSGL-1 expression was maintained on most LLO-specific CD4 T cells into memory (**Supplementary Figure S3b,c**). Of note, PSGL-1 expression levels correlated with expression of Ly6C as Ly6C^+ cells expressed higher levels of PSGL-1 and Ly6C^− cells expressed lower levels of PSGL-1 (**Supplementary Figure S3c**). During primary infection, the majority of the mucosal LLO-specific CD4 T cells that migrated into the intestinal effector sites upregulated CD69 and a small subset also upregulated CD103 in the IEL. However, most memory LLO-specific CD4 T cells failed to upregulate CD103 in the LP or IEL compartments demonstrating that CD103 is not responsible for the accumulation or retention of most LLO-specific CD4 T cells in intestinal tissues (**Figure 2e**). In contrast, most LLO-specific CD4 T cells in lymphoid tissues did not upregulate CD69. These data suggest that CD69 and PSGL-1 but not CD103 may mediate LLO-specific CD4 T-cell accumulation in the intestinal mucosa after oral infection. Collectively, these data establish that oral infection may have a substantial impact on the generation and maintenance of a distinct yet stable population of intestinal pathogen-specific CD4 T cells in intestinal tissues.

Intestinal mucosal memory CD4 T cells are predominantly Th1

It is well established that infection with *Lm* induces a robust Th1 response from responding *Lm*-specific CD4 T cells. However, many factors may impact CD4 T-cell functionality. For example, intranasal infection with *Lm* induces a population of CD27^− CD4 T cells that produce some IL-17A but are not maintained long-term.[6] Alternatively, CD27^− Th17 are maintained long term in conditions of chronic or intermittent antigenic stimulation, such as those of cancer or autoimmune models.[33] Moreover, the intestinal mucosa comprises a unique environment that may influence the function of memory CD4 T cells. As a population of LLO-specific CD27^− CD4 T cells were maintained long-term in the intestinal mucosa, we sought to identify whether these cells provided a long-lived source of memory cells capable of rapidly producing IL-17A to a secondary infection. Therefore, we examined LLO-specific
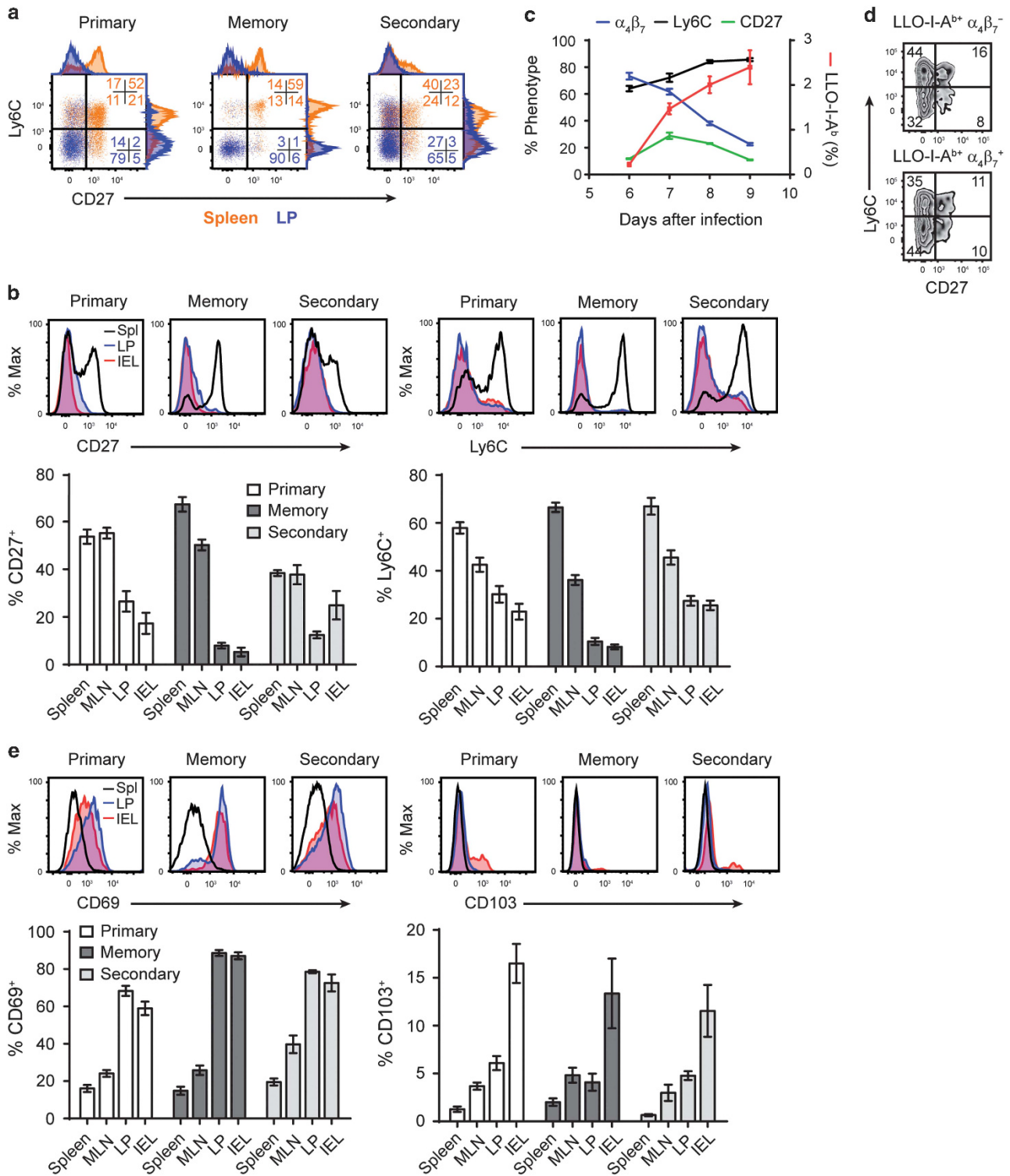
Figure 2 Intestinal CD4 T cells display distinct phenotypic characteristics. B6 mice were orally infected with InlA^M r*Lm* and LLO-specific CD4 T cells were analyzed for the indicated markers from the spleen, MLN, LP, and IEL compartment after infection. (**a**) Representative dot plots with adjunct histograms are gated on LLO-I-A^{b+} CD4 T cells at 9 (primary) and 60 (memory) dpi, or 7 days after a secondary challenge of mice that were immunized 60 days previously (secondary). For each dot plot, LLO-I-A^{b+} CD4 T cells from the LP (dark blue) are overlaid onto LLO-I-A^{b+} CD4 T cells from the spleen (light orange). Numbers within plots are the percentage of cells within gated quadrants and are color coded to the tissue they derive from. (**b**, **e**) Representative histograms are gated on live LLO-I-A^{b+} CD4 T cells at 9 (primary) and 60 (memory) d.p.i., or 7 days after a secondary challenge of mice that were immunized 60 days previously (secondary). The graphs display pooled data from 9 dpi (primary), between 50 and 90 d.p.i. (memory), and at 7 days after secondary infection of mice immunized 60 days previously (secondary). The graphs show the mean ± s.e.m. of pooled data from 2–7 independent experiments with 6–27 mice total. (**c**, **d**) LLO-specific CD4 T cells were enumerated from the blood at the indicated days after infection and examined for $\alpha_4\beta_7$, Ly6C, and CD27 expression. (**c**) Line graph depicts the mean ± s.e.m. of three mice per group and representative of two independent experiments. (**d**) Representative zebra plots from 7 d.p.i. mice are gated on LLO-I-A^{b+} CD4 T cells that do or do not express $\alpha_4\beta_7$.

effector and memory CD4 T cells in the small intestine after oral InlA^M rLm infection to determine whether maintenance in intestinal tissues modified the functional characteristics of intestinal memory CD4 T cells. LLO-specific CD4 T-cell effector functions were determined during primary infection, memory homeostasis and secondary challenge after ex $vivo$ LLO peptide stimulation. IFNγ producing CD4 T cells accounted for the majority of the T-cell response to LLO peptide examined during all phases of the immune response (**Figure 3a**). In addition, these cells were capable of simultaneously producing high levels of tumor necrosis factor-α (TNFα) and IL-2 (**Figure 3b,c**). Despite a demonstrable loss of functionality of CD4 T cells in the LP and IEL when compared with CD4 T cells in spleen, a large multifunctional population can be detected in the intestinal tissues. LLO-specific effector CD4 T cells capable of simultaneously producing IFNγ, TNFα, and IL-2 declined from 74% in the spleen to 58% in the LP, yet the overwhelming majority of LLO-specific CD4 T cells produced two or more cytokines simultaneously (94% in spleen compared with 89% in LP). Surprisingly, the LLO-specific CD4 T-cell population became less polyfunctional over time in both lymphoid and intestinal tissues. A substantial population of memory CD4 T cells producing IFNγ alone or together with IL-2 emerged in all tissues examined during memory homeostasis, but was particularly profound in the IEL compartment (**Figure 3b,c**). Memory cells producing IL-2 but not IFNγ were not readily detectable (data not shown) suggesting the emergence of Tem-like cells after oral Lm infection. In the intestines, IL-17A was largely produced by polyclonally stimulated CD4 T cells demonstrating that while some LLO-specific memory CD4 T cells could produce IL-17A, the majority of LLO-specific cells preferentially produced Th1 cytokines. Moreover, the small population of LP LLO-specific Th17 memory cells did not appear to contribute substantial IL-17A to the secondary response (**Figure 3a**), suggesting an inability to expand or a switch to IFNγ production. Thus, oral $Listeria$ infection predominately induced a long-lived population of CD27$^-$ CD4 T cells in the intestines that were functionally distinct with defined IFNγ and minimal IL-17A production.

Intestinal CD4 T-cell memory is IL-15 independent

The factors that regulate CD4 T-cell memory homeostasis have been less clear than for CD8 T cells. IL-15 is critical for the maintenance of certain T-cell subsets in intestinal tissues and memory CD8 T cells.[34] Although IL-15 is also important for pathogen-specific CD4 T-cell memory homeostasis it appears less critical than it is for CD8 T-cell memory homeostasis and is context dependent.[35] Moreover, recent evidence suggests the availability of mucosal niches where IL-15-independent maintenance of T-cell memory may occur.[36] We first tested whether LLO-specific CD4 T cells were influenced by the absence of IL-15. To examine this directly, WT or IL-15 knockout (KO) mice were orally infected with InlA^M rLM and LLO-specific CD4 T cells were examined in lymphoid and intestinal tissues over 180 days. Initially, a large defect

was observed in the percentage of LLO-specific CD4 T cells in the lymphoid compartment but not intestinal effector sites at 9 d.p.i. (**Figure 4a**). However, the absolute number of LLO-specific CD4 T cells was similar between WT and IL-15 KO mice in all tissues examined at 9 d.p.i. (**Figure 4b**). These results suggest that IL-15 is dispensable in regulating the magnitude of the pathogen-specific effector CD4 T-cell population. In addition, LLO-specific memory CD4 T cells were maintained at least comparably in IL-15 KO mice at each time point examined after T–cell contraction regardless of tissue location (**Figure 4a,b**). Consistent with this observation memory LLO-specific CD4 T cells did not express appreciable levels of the common IL-2 and IL-15 receptor β subunit, CD122, but did express high levels of IL-7 receptor α, CD127 (**Supplementary Figure S4a**). Moreover, LLO-specific memory CD4 T cells from IL-15 KO mice were phenotypically similar to LLO-specific memory CD4 T cells from WT mice based on expression of CD69, CD103, CD27, and Ly6C (**Supplementary Figure S4b,c**). Collectively, these data suggest that memory LLO-specific CD4 T cells are maintained independently of IL-15 after oral Lm infection. However, it should also be noted that the number of memory IL-15-deficient LLO-specific CD4 T cells eventually outpaced the number of WT LLO-specific CD4 T cells in lymphoid tissues.
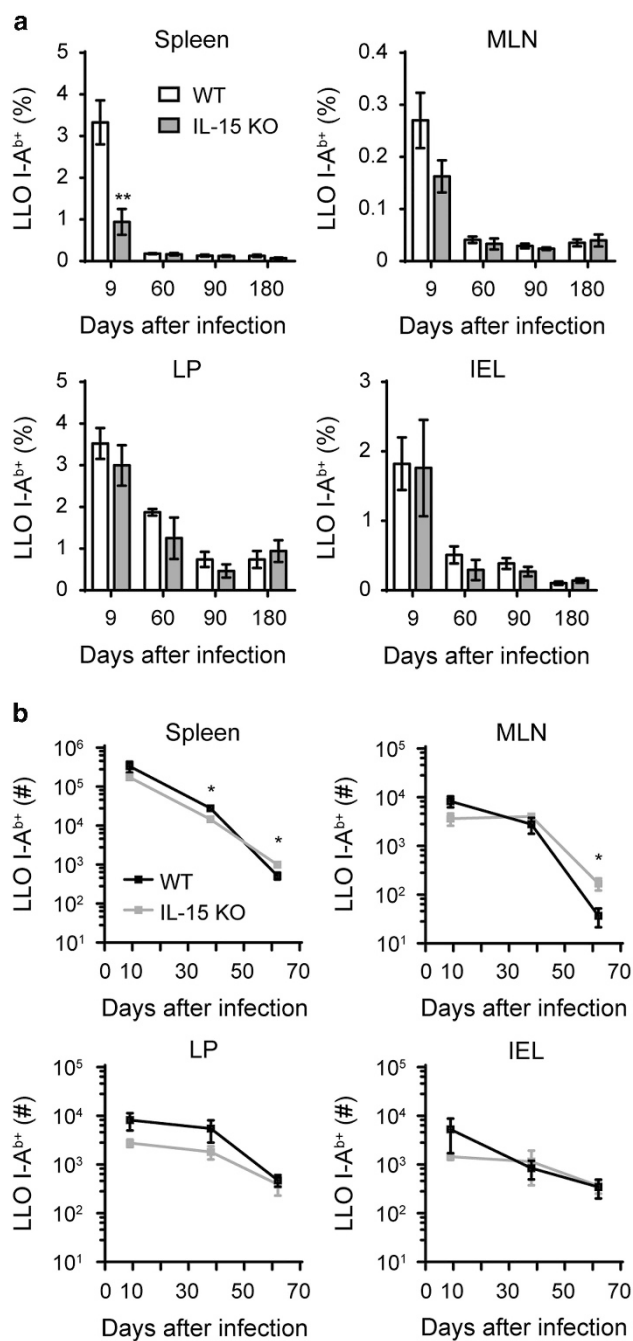
CD4 T cells contribute to protection from challenge infection

CD4 and CD8 T cells have an important role in limiting bacterial growth and dissemination after secondary intravenous Lm infection.[37] We hypothesized that mucosal memory CD4 T cells participate in the protection of the intestinal mucosa. CD4 T-cell help is critical during priming of the CD8 T-cell response to induce efficient CD8 effectors after a secondary challenge.[38] However, CD4 T-cell depletion has little effect on the function and protective capacity of CD8 T-cell response to Lm if CD4 T-cell depletion only occurs during the secondary response.[37,39,40] We evaluated whether anti-CD4 (clone GK1.5) treatment impacted CD4 T-cell numbers and memory CD8 T cells in intestinal tissues after oral InlA^M rLm secondary infection. First, anti-CD4 treatment led to an ∼90% reduction in CD4 T cells in all tissues examined (**Supplementary Figure S5a**). In addition, no detrimental effect of CD4 T-cell depletion could be observed on ova-specific CD8 T cells 2 days after challenge infection (**Supplementary Figure S5a**). Therefore, we used antibody depletion of CD4 T cells during secondary oral InlA^M rLm infection to determine the contribution of CD4 T cells to mucosal protection early after challenge. Immunized mice were treated with anti-CD4 prior to and shortly after rechallenge with InlA^M rLm and the bacterial burden was quantified in the small intestine and MLNs 4 days later. Elevated listeria burden was observed in the intestines and MLN of anti-CD4 antibody treated mice (**Figure 5**) suggesting that memory CD4 T cells have an important role in mucosal protection against intracellular pathogens in the intestine. As circulating CD4 T cells may contribute to the protection observed in the intestines 4 days
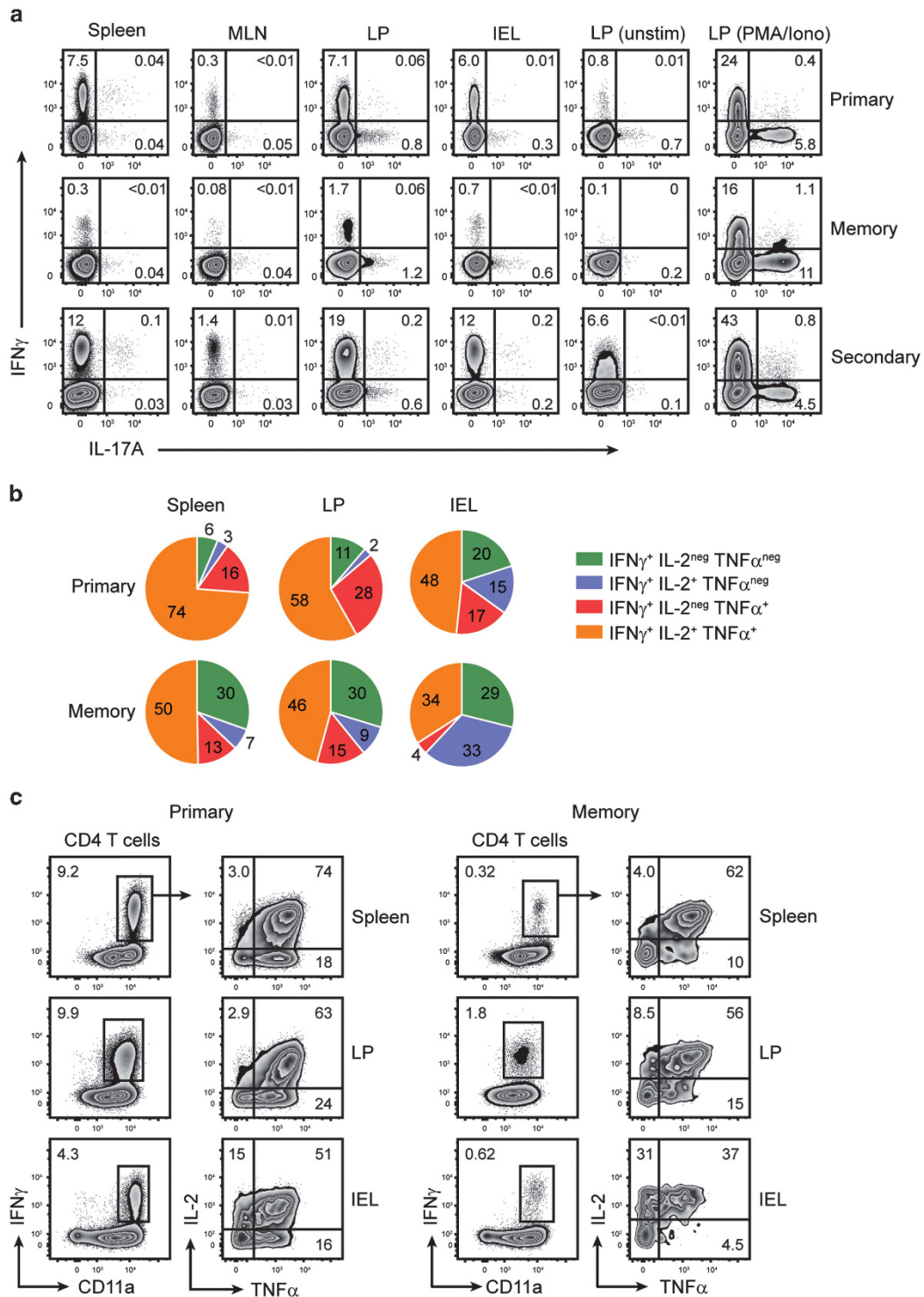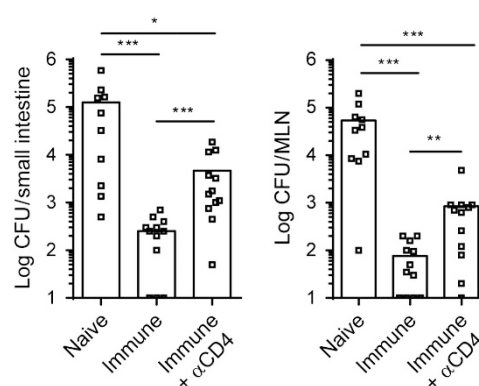
Figure 3 Polyfunctional LLO-specific CD4 Th1 cells in intestinal tissues. Single-cell suspensions isolated from the spleen, MLN, LP, or IEL during the primary (9 d.p.i.), memory (60 d.p.i.), or secondary (60 + 7 d.p.i.) responses were stimulated with $LLO_{190-201}$ peptide in the presence of brefeldin A, with BD leukocyte activation cocktail (PMA/Iono), or with brefeldin A alone (unstim). (**a**) $IFN\gamma$ and IL-17A expression was determined by intracellular cytokine staining after 5 h at 37 °C and 5% CO_2. Representative zebra plots are gated on live $CD45^+ CD4^+$ T cells and numbers within quadrants correspond to the percentage of cells within gates. Data are representative of 3 experiments with at least three mice per group. (**b**) Pie charts display the multifunctional nature of CD4 T cells. Multifunctionality was determined among an $IFN\gamma^+ CD4^+$ parent population. Numbers in pie charts represent the mean percentage of $IFN\gamma^+$ cells that produced the indicated cytokines. Data depict the mean of three mice per group and are representative of two experiments. (**c**) Representative zebra plots are presented to demonstrate the multifunctional CD4 T-cell response. Numbers within gates represent the mean percentage of concatenated cells that produced the indicated cytokines and are representative of two experiments.

Figure 5 Memory CD4 T cells provide protection from secondary oral InlA^M rLm infection. Naive or immunized mice were challenged with oral InlA^M rLm infection. Some immunized mice were also treated with anti-CD4 (clone GK1.5) to deplete CD4 T cells. The small intestines and MLN were collected 4 days after challenge infection and Lm burden was quantified on agar plates supplemented with streptomycin. Pooled data from two individual experiments with five mice per experiment are shown. The bar graph depicts the mean and individual mice are shown for each group. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ (Mann–Whitney test).

Figure 4 Maintenance of CD4 memory is IL-15 independent. B6 wild-type (WT) and IL-15 deficient (IL-15 knockout (KO)) mice were orally infected with InlA^M rLm and LLO-specific CD4 T-cell populations were examined in the spleen, MLN, LP, and IEL for frequency (**a**) and number (**b**) at the indicated times after infection. Data are representative of two to four individual time course experiments with three to eight mice per time point and depict the mean ± s.e.m. *$P<0.05$, **$P<0.01$ (unpaired Student's t-test).

contributes to protective memory independent of effects on the memory CD8 compartment.

DISCUSSION

Although our understanding of how CD4 T cells differentiate into functionally distinct subsets has been substantially advanced over the last decade, the persistence of memory CD4 T cells and the factors that regulate the functional properties of memory CD4 T cells remains incompletely understood. An even greater dearth of knowledge is evident when examining peripheral and mucosal CD4 T-cell populations, such as those maintained long term in the intestinal mucosa after infection. Here we have utilized an InlA^M rLm oral feeding infection model to demonstrate the substantial differences in antigen-specific memory CD4 T-cell populations in intestinal tissues after oral infection with an enteric pathogen. In this context, memory CD4 T cells in intestinal mucosa were phenotypically distinct and were maintained independent of IL-15. Intestinal LLO-specific CD4 T cells displayed a distinct Trm phenotype with polyfunctional Th1 cytokine properties. In addition, we demonstrate that CD4 T cells provided substantial protection upon reinfection with oral InlA^M rLm and this effect may be independent of the pathogen-specific CD8 T-cell response. These findings highlight the importance of understanding CD4 T-cell responses to bacterial pathogens in the intestinal mucosa and their potential to serve as a platform to design novel strategies for vaccine development as well as to identify targeted therapies for intestinal tumors and inflammatory diseases.

CD4 T cells are the most abundant T-cell in the LP of the intestinal mucosa. The imprinting of CD4 T cells to home to infected tissues predominantly occurs at the induction site of the immune response. Consistent with intestinal priming in MLN or GALT, circulating LLO-specific CD4 T cells expressed high levels of the gut homing integrin $\alpha_4\beta_7$ early after infection

after challenge infection, bacterial burdens were also assessed 2 days after challenge infection. Increased bacterial burdens were observed in the small intestine and MLNs of anti-CD4 treated mice (**Supplementary Figure S5b**). Collectively, these data suggest that early CD4-mediated control in the intestinal tissues

that was rapidly downregulated. Similar to CD8 T-cell migration, these data suggest the presence of a narrow window of effector CD4 T-cell migration into intestinal tissues. In this regard, CD4 Trm seeding of intestinal tissues likely occurs during initial migration of a population composed of terminally differentiated effectors and long-lived cells. Interestingly, LLO-specific CD4 T cells also accumulated extensively in the IEL compartment, an environment where large numbers of CD4 (CD8α$^-$) T cells are not typically found. Thus, the protective role CD4 T cells provided upon oral Lm infection highlight the importance of pathogen-specific CD4 T cells in protecting intestinal mucosa against intracellular pathogens. It is likely that several factors impact the ability of pathogen-specific CD4 T cells to provide protection against secondary infections. First, there is a large number of Lm-specific CD4 T cells in the LP and IEL compartments to serve as sentinels for reinfection. Trm provide specific and immediate functions and they also mediate the recruitment of innate and adaptive arms of the immune response to help clear invading pathogens. Lm-specific CD4 T cells produce large amounts of IFNγ, which has recently been identified as a key mediator of Trm alarm functions.[41] Effector cytokines produced by intestinal LLO-specific CD4 memory cells may drive accelerated recruitment of innate mediators such as monocytes and neutrophils important for clearance of Listeria. In addition, a small population of LP-resident LLO-specific CD4 T cells express granzyme B and may possess CTL capabilities. Finally, CD4 T cells may provide help to CD8 CTL by facilitating accumulation or retention in intestinal tissues.[42,43] While the number of Lm-specific CD8 memory T cells was not negatively impacted following CD4 T-cell depletion prior to and during a secondary challenge infection, the mechanism of CD4 T-cell protection in the intestinal tissue is likely multifaceted and a more thorough investigation of the mechanism of CD4 T-cell protection in the intestinal mucosa is warranted.

The maintenance of memory T cells has traditionally been considered dependent on the presence of IL-15 though recent evidence indicates some IL-15-independent niches exist.[36] In the case of the intestinal mucosa, the presence of IL-15 is critical for the maintenance of CD8αα$^+$ γδ and αβ T cells in the IEL compartment.[34] The specific context of pathogen-specific CD4 T cells also appears to dictate the requirement for IL-15 for maintenance.[35] In this regard, the maintenance and phenotype of intestinal pathogen-specific memory CD4 T cells does not appear to be regulated by IL-15 after oral InlA^M rLm infection as comparable populations of LLO-specific CD4 T cells were maintained in the IEL and LP compartments of IL-15-deficient mice following resolution of infection. Although LLO-specific CD4 T cells do not express CD122, they express high levels of the IL-7 receptor α chain CD127 suggesting that IL-7 may regulate intestinal memory populations. Collectively, these data suggest a dynamic regulation of pathogen-specific memory cells and their potential precursors for the intestinal CD4 T-cell compartment.

Several markers have been described to analyze pathogen-specific CD4 T-cell subsets predisposed for memory formation.

In our study, we evaluated putative memory CD4 T-cell markers and examined their relationship between lymphoid and intestinal compartments after oral InlA^M rLm infection. Using CD27 and Ly6C, we observed differences between peripheral and lymphoid LLO-specific CD4 T cells. During the peak of infection, the majority of LLO-specific effector CD4 T cells in intestinal tissues did not express CD27 or Ly6C. During effector phases of the immune response a small subset of Ly6C$^+$ and KLRG1$^+$ cells emerged in the intestinal tissues but these populations were lost during resolution of the immune response. It is unclear whether these cells underwent apoptosis or downregulated these markers. Similar to KLRG1$^+$ CD8 T cells in intestinal tissues,[19] KLRG1$^+$ CD4 T cells may not survive well after emigration into the intestinal environment. On the other hand, LLO-specific CD4 T cells found in the spleen and MLNs had heterogeneous populations of CD27 and Ly6C expressions that remained predominantly stable throughout all phases of the immune response. Thus, we have identified a phenotypically distinct and protective CD4 T-cell response in the intestinal mucosa after oral infection.

Maintenance of CD8 T cells in intestinal mucosa has also been associated with transforming growth factor-β-induced CD103 expression and its interaction with epithelial expressed E-cadherin.[26,44,45] However, most CD4 T cells in peripheral tissues do not express CD103 and several studies indicate CD103-independent retention mechanisms exist. Consistent with these observations, most intestinal LLO-specific CD4 T cells did not express CD103 after oral InlA^M rLm infection suggesting that the accumulation and maintenance of pathogen-specific CD4 IELs may be independent of CD103. CD4 T cells residing in mucosal tissues express CD69 on their surface, a molecule that inhibits S1P1 signals,[46] a key requirement to establish residency.[31] In our observations, a majority of LLO-specific CD4 T cells from IEL and LP compartments but not the lymphoid tissues expressed CD69, suggesting that LLO-specific CD4 T cells are resident in intestinal mucosa. Nevertheless, key factors that promote pathogen-specific CD4 T-cell survival and retention in the intestinal mucosa requires further elucidation.

In our studies, LLO-specific CD4 T cells displayed a strong polyfunctional Th1 profile when stimulated ex vivo. Contrary to LLO-specific CD8 T cells after oral InlA^M rLm infection,[19] LLO-specific CD4 T cells appear less multifunctional then their lymphoid counterparts during the primary and memory phases. A substantial population of CD4 T cells producing IFNγ alone or with IL-2 emerges in intestinal tissues at memory suggesting Tem-like cells develop in all tissues.[13] Despite the overall reduction in multifunctionality, ∼50% or more of responding CD4 T cells were capable of producing IFNγ, IL-2, and TNFα simultaneously in the spleen and LP. CD4 T cells are also a dominant source of IL-2 in peripheral mucosal tissues of humans[11] suggesting that a similar dichotomy exists in the intestinal mucosa after oral infection of mice. The additional observation of a small subset of memory CD4 T cells capable of producing IL-17A warrants further evaluation into mechanism of lineage commitment in the intestinal mucosa.

In conclusion, oral infection with an intracellular bacterial pathogen illustrates (1) the induction of protective CD4 T-cell responses in the intestinal mucosa, (2) the formation and maintenance of intestinal CD4 T-cell memory, (3) the functional and phenotypic characteristics, which highlight a distinct intestinal pathogen-specific CD4 T-cell response, and (4) the recall and protective capacity of endogenous pathogen-specific CD4 T cells to secondary intestinal infections. These results advance our understanding of CD4 T-cell responses to pathogens in intestinal tissues and highlight the distinct impact of the intestinal compartment on generation of pathogen-specific CD4 T cells.

METHODS

Mice and infection. C57BL/6 mice were purchased from the National Cancer Institute and The Jackson Laboratory. IL-15 KO mice were generously provided by Dr Jacques Peschon (Immunex, Seattle, WA). LLO56 TCR transgenic mice were generously provided by Dr Paul Allen (Washington University, St Louis, MO). All animal protocols were in accordance with the IACUC guidelines and approved by the University of Connecticut Health Center and Stony Brook University Institutional Animal Care and Use Committees. Mice were food and water deprived for 4 h prior to infection. Individually housed mice were fed bread containing 2×10^9 or 2×10^{10} CFU InlA^M rLm (strain EGDe or 10403s) for primary or secondary infection. In some cases, mice were infected with InlA^M rLm expressing truncated ovalbumin (rLm-ova).

Lymphocytes isolation and flow cytometry. Single-cell suspensions were isolated from collagenase digested or mechanically dispersed MLNs and spleens. Cells from LP and IEL compartments were isolated as previously described.[19] Cells ($5-10 \times 10^6$) were stained with LLO$_{190-201}$ I-A^b streptavidin–phycoerythrin (PE) or LLO$_{190-201}$ I-A^b streptavidin–allophycocyanin MHC class II tetramers in IMDM medium supplemented with 10% fetal bovine serum and 50 nM Dasatinib for 1 h at 37 °C. LLO I-A^b tetramers were kindly provided by Dr Marc Jenkins (University of Minnesota, Minneapolis, MN). In some experiments, tetramer enrichment was used for quantification of LLO-specific CD4 T cells. In these experiments, cells were further incubated with anti-PE or anti-allophycocyanin magnetic beads (Miltenyi Biotec, Auburn, CA) for positive selection through a magnetic column. Ova$_{257-264}$ H-2K^b streptavidin –allophycocyanin MHC class I tetramers were made from MHC class I monomers provided by the NIH Tetramer Core Facility. MHC I tetramer staining was performed at room temperature for 1 h. Surface antigens were then identified with antibodies for 30 min at 4 °C.

Intracellular cytokine staining. Cells ($5-10 \times 10^6$) isolated from spleens, MLN, LP, or IEL compartments were incubated with 1 µg ml^{-1} Golgiplug (BD Biosciences, San Jose, CA) with or without 10 µg ml^{-1} LLO-derived peptide NEKYAQAYPNVS (LLO$_{190-201}$) or with leukocyte activation cocktail (BD Pharmigen, San Jose, CA) for 5 h at 37 °C and 5% CO$_2$. IELs were also incubated with congenic mismatched, naïve splenocytes. Cells were then stained for surface markers for 30 min at 4 °C. Cells were treated with BD Cytofix/Cytoperm (BD Biosciences) for 20 min at 4 °C prior to intracellular staining with anti-IFNγ, anti-IL-2, anti-IL-17A, and anti-TNFα.

Enumerating bacterial burden. Infected mice were treated with 200 µg GK1.5 (BioXcell, West Lebanon, NH) intraperitoneally at day −3, −1, and +1 respective to challenge infection. Control mice were treated with 200 µg LTF-2. Tissues were harvested 2 and 4 days post challenge for enumeration of bacterial burden. Single-cell suspensions from the MLNs were incubated with 1% saponin (Calbiochem, Billerica, MA) for 1 h at 4 °C. Intestines were prepared using gentleMACS dissociator (Miltenyi Biotec) and then incubated with 1% saponin for 1 h at 4 °C. Supernatants were plated on brain heart infusion agar containing 50 µg ml^{-1} streptomycin at 37 °C. Bacterial colonies were enumerated 24–48 h later.

Confocal microscopy. Small intestines were fixed overnight in paraformaldehyde-lysine-periodate solution (PLP) as described previously.[47] Briefly, sections of small intestines were fixed in PLP solution (0.05 M phosphate buffer, 0.1 M L-lysine, 2 mg ml^{-1} NaIO$_4$ and 4% paraformaldehyde), washed extensively with phosphate-buffered saline and dehydrated in 30% sucrose. Tissues were snap frozen in OCT compound (Tissue-Tek) and 30-µm frozen sections were cut using a cryostat (Leica CM1850). Sections were fixed with ice-cold acetone for 10 min, and air-dried for 30 min and then incubated with 0.3 M Glycine for 30 min and with 5% normal goat serum, 5% fetal bovine serum and anti-CD16/32 in phosphate-buffered saline for 45 min. Sections were stained for primary antibodies for 1.5 h at room temperature, washed and stained for secondary or fluorophore-conjugated antibodies and DAPI for 1 h at room temperature. Images were acquired using an LSM 780 (Carl Zeiss, Oberkochen, Germany) and images were processed using Imaris (Bitplane, Belfast, UK).

Statistical analysis. Statistical significance was calculated with GraphPad Prism (GraphPad Software, La Jolla, CA) using an unpaired two-tailed t test or Mann–Whitney test as indicated.

DISCLOSURE
The authors declared no conflict of interest.

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