$CD4^+$ T help promotes influenza virus-specific $CD8^+$ T cell memory by limiting metabolic dysfunction

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Contributed by Peter C. Doherty, January 10, 2019 (sent for review May 23, 2018; reviewed by Ashraful Haque and Kim Klonowski)

There is continued interest in developing novel vaccine strategies that induce establish optimal $CD8^+$ cytotoxic T lymphocyte (CTL) memory for pathogens like the influenza A viruses (IAVs), where the recall of IAV-specific T cell immunity is able to protect against serologically distinct IAV infection. While it is well established that $CD4^+$ T cell help is required for optimal CTL responses and the establishment of memory, when and how $CD4^+$ T cell help contributes to determining the ideal memory phenotype remains unclear. We assessed the quality of IAV-specific $CD8^+$ T cell memory established in the presence or absence of a concurrent $CD4^+$ T cell response. We demonstrate that $CD4^+$ T cell help appears to be required at the initial priming phase of infection for the maintenance of IAV-specific CTL memory, with "unhelped" memory CTL exhibiting intrinsic dysfunction. High-throughput RNA-sequencing established that distinct transcriptional signatures characterize the helped vs. unhelped IAV-specific memory CTL phenotype, with the unhelped set showing a more "exhausted T cell" transcriptional profile. Moreover, we identify that unhelped memory CTLs exhibit defects in a variety of energetic pathways, leading to diminished spare respiratory capacity and diminished capacity to engage glycolysis upon reactivation. Hence, $CD4^+$ T help at the time of initial priming promotes molecular pathways that limit exhaustion by channeling metabolic processes essential for the rapid recall of memory $CD8^+$ T cells.

$CD8^+$ T cell | immunological memory | CD4 T cell | influenza | metabolism

It is well accepted that the activation of $CD4^+$ T helper cells is key for ensuring the maturation of protective humoral and cellular immunity following pathogen challenge. Even so, when it comes to generating effective cytotoxic T lymphocyte (CTL) responses in naïve individuals, the need or otherwise, for $CD4^+$ T cell involvement is highly dependent on the nature of the immune challenge. For example, $CD4^+$ T cell-independent primary CTL effectors can be readily induced in the context of robust acute viral (1–3) or bacterial infections that induce a strong inflammatory response (4). In contrast, the acute response to immunogens that induce low levels of inflammation looks to be more $CD4^+$ T cell help-dependent (2, 5).

Beyond the primary CTL response, the precise role $CD4^+$ T help in the establishment of optimal $CD8^+$ T cell memory after immunization or infection remains less clear. Initial work suggested that regardless of whether the primary CTL response was $CD4^+$ T cell-dependent or -independent, $CD4^+$ T help during the initial priming phase was necessary for the generation of memory T cells capable of responding to secondary challenge (2, 3). The proposed mechanism is that these helpers induced, at least in a subset of activated CTLs, molecular profiles that ensure optimal $CD8^+$ T cell memory (6). Such programming likely reflects augmented signaling from cytokines, such as IL-2 (7), and the delivery of costimulatory signals that promote dendritic cell (DC) activation (5) to ensure that, at least for some CTL precursors (CTLps), pathways that regulate T cell survival are engaged (8–10). Furthermore, there is evidence that $CD4^+$ T cell help is also needed for memory CTLp maintenance, with elimination of the $CD4^+$ set after priming, resulting in gradual loss of memory $CD8^+$ T cell numbers and function (11).

As observed with primary CTL responses, memory CTL formation in certain circumstances can also be largely independent of $CD4^+$ help, as observed after vesicular stomatitis virus (11) or ectromelia (mousepox) virus infection (12). Moreover, the extent of $CD4^+$ T cell-dependence for the establishment of CTL memory can also vary for responses targeted to different peptides from the same immunogen (13). Utilizing a mouse model influenza A virus (IAV) infection, it has been previously demonstrated that primary IAV-specific CTL responses are largely independent of $CD4^+$ T cell help (1, 14). Overall, these studies highlight that, in a manner similar to primary CTL responses, $CD4^+$ T help-dependence for establishing effective CTL memory is context-dependent. Even so, many gaps remain in our understanding of both the necessary timing and the underlying molecular mechanisms of IAV-specific memory CTL formation.

The present analysis utilizes an adoptive transfer model to further probe the necessity for $CD4^+$ T cell help in the establishment of enduring IAV-specific $CD8^+$ T cell memory, then uses RNA-sequencing (RNA-seq) analysis to dissect the molecular pathways characteristic of the "helped" vs. "unhelped" memory CTL sets. Our data suggest that $CD4^+$ T cell-dependent

Significance

Promoting effective $CD8^+$ T cell memory is a primary goal of T cell-based vaccination and immunotherapy strategies. While it is well established that $CD4^+$ T cell help is required for enduring $CD8^+$ T cell memory, how such help contributes to establishing optimal $CD8^+$ T cell memory generation and persistence remains unclear. In this study, we demonstrate that $CD4^+$ help at the time of priming ensures that memory $CD8^+$ T cells are programmed to engage metabolic biological pathways essential for effective recall responses. Such understanding has clear implications for the augmentation of vaccine therapies designed to promote protective T cell immunity.

Author contributions: J.G.C., H.A.M., B.E.R., N.L.L.G., and S.J.T. designed research; J.G.C., H.A.M., K.M.Q., M.O., B.E.R., A.M., S.W., J.E.P., and S.J.T. performed research; J.G.C., H.A.M., K.M.Q., M.O., B.E.R., A.M., S.W., J.E.P., N.L.L.G., P.C.D., and S.J.T. analyzed data; and J.G.C., H.A.M., N.L.L.G., P.C.D., and S.J.T. wrote the paper.

Reviewers: A.H., QIMR Berghofer Medical Research Institute; and K.K., University of Georgia.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1808849116/-/DCSupplemental.

Published online February 20, 2019.

programming at the time of initial priming engages the appropriate molecular pathways required to limit $CD8^+$ T cell exhaustion and, as a consequence, ensures the rapid recall of IAV-specific CTLs from the memory pool following pathogen challenge. These data thus provide insights into how $CD4^+$-dependent CTL memory is regulated and have implications for developing vaccination strategies, with the potential to promote a measure of protection against a novel IAV pathogen.

Results

$CD4^+$ T Cell Help Is Required During the Initial $CD8^+$ T Cell Priming Phase. Primary IAV-specific CTL responses are $CD4^+$ T cell-independent, while the establishment of functional IAV-specific CTL memory requires a concurrent $CD4^+$ T cell response (1, 14, 15). Whether $CD4^+$ T cell help is required at the time of priming, or is required for memory CTL maintenance, is not clear. To address this question, we utilized an adoptive transfer model (16), whereby 1×10^4 naïve ($CD62L^{hi}CD44^{lo}$), congenically marked (CD45.1) OT-I $CD8^+$ T cells, specific for the ovalbumin peptide ($OVA_{257–264}$), were transferred into either wild-type C57BL/6 (helped) or CD4-deficient (unhelped) GK1.5 transgenic (Tg) (17), mice. Mice that received naïve OT-I $CD8^+$ T cells were then infected intranasally with the recombinant A/HKx31-OVA virus (18) to induce an OT1-specific CTL response. As previously reported for endogenous IAV-specific CTLs (1), primary OT-I CTL generation was equivalent in both the presence and absence of $CD4^+$ T cells (Fig. 1). However, there were significantly fewer OT-I–specific resting memory CTLps in unhelped vs. helped mice (Fig. 1 and *SI Appendix*, Fig. S1*A*). This highlighted that $CD4^+$ T cell help was required, either during priming or maintenance, to generate an optimal IAV-specific memory CTL population (19). To examine the impact of a lack of $CD4^+$ T cell help on OT-I–specific CTL recall responses, memory mice were challenged with the serologically distinct A/PR8-OVA virus (Fig. 1). In this case, unhelped memory CTLs did not expand to the same extent as helped memory CTL. The decreased secondary response exhibited by unhelped memory OT-I CTLs was not just due to fewer memory precursors, because helped memory CTLs proliferated to a much greater extent (37-fold vs. 6-fold) (Fig. 1*B*). This indicated that unhelped memory OT-I CTLs potentially exhibit an intrinsic defect in recall capacity.

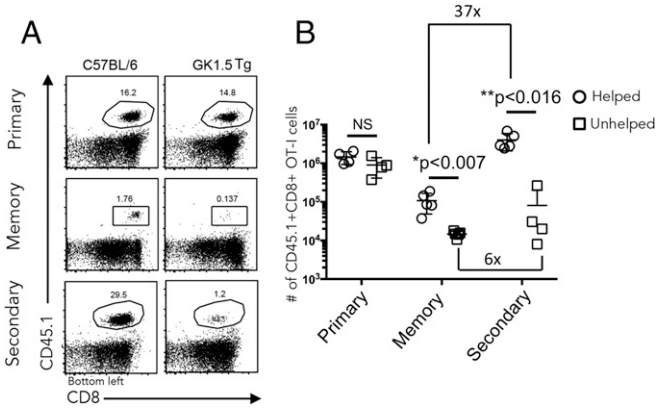

Fig. 1. Compromised memory and secondary recall responses in GK1.5Tg mice. Congenic (CD45.1), naïve ($CD44^{lo}CD62L^{hi}$) OT-I T cells (10^4) were adoptively transferred into either uninfected B6 or GK1.5Tg mice (CD4 depleted) followed by intranasal infection with 10^4 pfu of A/HKx31-OVA (18). (*A*) Congenic (CD45.1), naïve ($CD44^{lo}CD62L^{hi}$) OT-I T cells (10^4) were adoptively transferred into either uninfected B6 (helped, *Top* and *Bottom*) or GK1.5Tg mice (unhelped, *Middle*) followed by intranasal infection with 10^4 pfu of A/HKx31-OVA (18). Helped (wild-type) memory (day 28) OT-I CTL were sort-purified and equal numbers transferred into either wild-type B6 (helped → helped; *Top*) or GK1.5Tg (helped → unhelped; *Bottom*) mice. Similarly, unhelped memory CTL were also transferred into wild-type B6 recipients (unhelped → helped, *Middle*). Shown are representative flow cytometry plots. (*B*) OT-I CTL were enumerated at day 10 (primary), day 28 (memory) or 8 d after secondary intranasal challenge with 100 pfu of A/PR8-OVA (secondary) from wild-type (○) or GK1.5Tg (□) recipients. Data shown mean ± SD. are representative of three independent repeats. Fold expansion was determined by dividing the secondary T cell numbers with memory T cell numbers. Significance tested with one-way ANOVA test with Tukey posttest (*$P < 0.007$; **$P < 0.016$; NS, not significant).

To better define whether the requirement for $CD4^+$ T help was necessary at either the time of priming or during the maintenance phase for IAV-specific CTL memory, we modified our adoptive transfer protocol to first establish OT-I–specific CTL memory in either wild-type B6 (helped) (Fig. 2*A*) or $CD4^+$ T cell-deficient GK1.5Tg (unhelped) (Fig. 2*A*) recipients. Equal numbers of HK-OVA–primed (10^4–10^5) helped or unhelped donor memory cells (day 28) were harvested and then transferred into naïve B6 mice, where $CD4^+$ T cell help is intact (Fig. 2*A*). Both the proportion (Fig. 2*B*) and absolute numbers (Fig. 2*C*) of OVA-specific memory CTLs within the spleen were enumerated following intranasal challenge with the HK-OVA virus. In this case, unhelped memory CTLs failed to expand to the same extent as helped memory CTLs (Fig. 2*C*). Interestingly, there was no measurable difference in cytokine production following in vitro restimulation with OVA peptide (*SI Appendix*, Fig. S1 *B* and *C*). The above results indicate that $CD4^+$ T cell help is critical for optimal memory CTL generation and recall. However, whether an autonomous helped profile is imprinted in the memory CTLps or requires, additionally, a concurrent $CD4^+$ T cell response following secondary challenge was not clear. To further clarify this issue, equal numbers (10^4) of helped (day 28) $CD8^+$ T cells, were adoptively transferred into GK1.5Tg mice, which were then challenged again with the HK-OVA virus (Fig. 2*A*, *Lower*). These helped CTLps responded equally in the CD4-intact and CD4-deficient recipients (Fig. 2 *B* and *C*). Collectively, these data demonstrate that, while the primary CTL effector response to IAV infection are $CD4^+$ T cell–independent, optimal CTL memory generation, and expansion requires $CD4^+$ T cell help at the time of priming and is not strictly required for memory CTL reactivation.

$KLRG1^{hi}$ $IL\text{-}7R^{lo}$ Short-Lived Effector Cells Are More Prominent in the Unhelped Memory CTLp Set. Patterns of cell surface KLRG1 and IL-7R expression are considered to identify distinct memory CTLp populations with short-lived effector cells (SLECs), characterized as $KLRG1^{hi}$ $IL\text{-}7R^{lo}$, and memory precursor cells (MPECs), characterized as $KLRG1^{lo}$ $IL\text{-}7R^{hi}$ (20, 21) (*SI Appendix*, Fig. S2). To determine the impact of $CD4^+$ T cell deficiency on the formation of IAV-specific SLEC and MPEC memory subsets, we assessed KLRG1 and IL-7R profiles for both helped vs. unhelped effector and memory OT-I CTLps following primary virus challenge (Fig. 3 *A* and *B*). The presence or absence of concurrent $CD4^+$ T help made no difference to the proportion of effector $CD8^+$ OT-I CTLs exhibiting SLEC ($KLRG1^{hi}IL7R^{lo}$) or MPEC ($KLRG1^{lo}IL7R^{hi}$) phenotypes at the peak of the primary response (Fig. 3*A*). However, for established memory, the SLEC ($KLRG1^{hi}IL7R^{lo}$) set was much more prominent in the unhelped CTLps (Fig. 3*B*). Therefore, in the absence of concurrent help, it thus seems that a higher proportion of the responding OT-I–specific CTLps are driven to a more terminally differentiated $KLRG1^{hi}$ $IL\text{-}7R^{lo}$ SLEC phenotype.

Unhelped and Helped Memory IAV-Specific CTLps Exhibit Distinct Transcriptional Profiles. To gain further molecular insights into why unhelped memory CTL failed to exhibit optimal recall capacity, we compared global transcriptional profiles for helped and unhelped CTLs isolated at either the peak of the primary response (day 10) (*SI Appendix*, Fig. S3*A*) or at a memory time

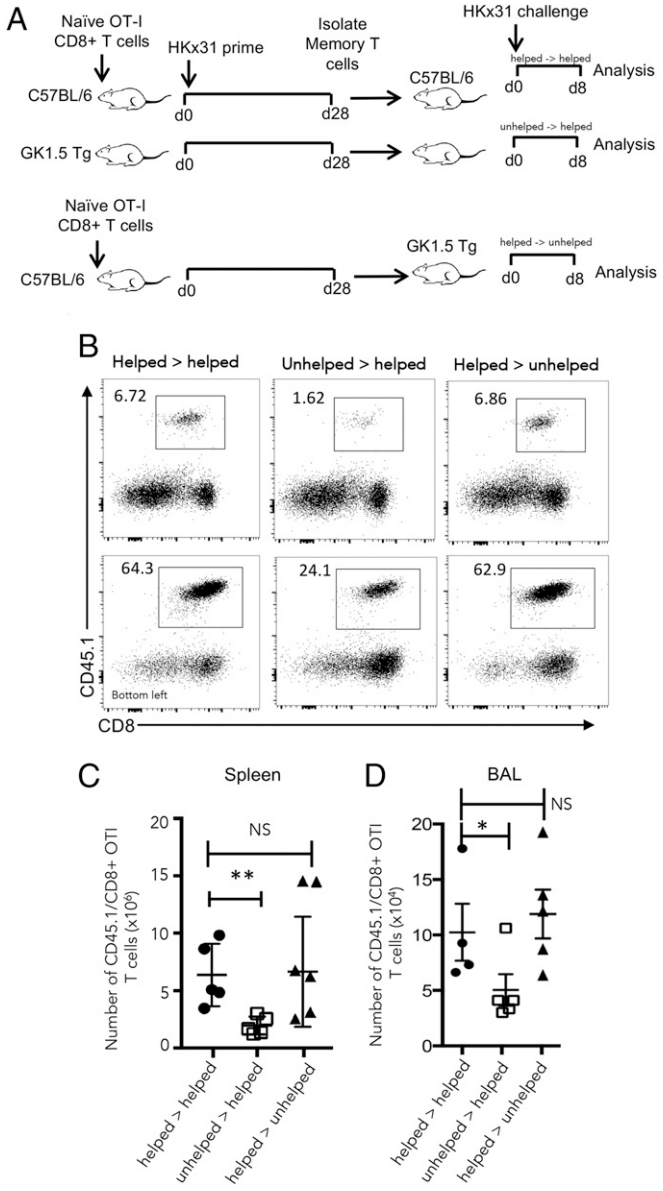

Fig. 2. CD4$^+$ T cell help is required at the time priming to establish memory recall capacity. (*A*) Congenic (CD45.1), naïve (CD44^{lo}CD62L^{hi}) OT-I T cells (10^4) were adoptively transferred into either uninfected B6 (helped, *Top*) or GK1.5Tg mice (unhelped, *Middle*) followed by intransal infection with 10^4 pfu of A/HKx31-OVA (18). Helped (wild-type) memory (day 28) OT-I CTL were sort purified and equal numbers transferred into either wild-type B6 (helped → helped; *Top*) or GK1.5Tg (helped → unhelped; *Middle*) mice. Similarly, unhelped memory CTL were also transferred into wild-type B6 recipients (unhelped → helped, *Bottom*). Splenocytes were isolated and stained for CD45.1 and CD8 to detect the proportion of OT-I T cells at day 8 after A/HKx31OVA challenge (*B–D*). (*B*) Shown are representative flow cytometry plots. (*C* and *D*) The number of helped → helped (●), unhelped → helped (□), and helped → unhelped (▲) OT-I CTL isolated from the spleen (*C*) or BAL (*D*), was determined 8 d after secondary challenge. Data shown mean ± SD and are representative of three independent repeats. Significance tested with one-way ANOVA test with Tukey posttest (**$P < 0.001$; *$P < 0.01$; NS, not significant).

point (day 28). Given that we had established that CD4$^+$ T cell help is required at the time of initial priming, we first examined transcriptional differences at day 10 before and after in vitro peptide stimulation. Principal component analysis (PCA) highlighted extensive overlap between helped and unhelped effector CTL differentially expressed genes (DEGs) (*SI Appendix*, Fig. S3*A*), with peptide stimulation resulting in much more significant differences than the presence or absence of CD4$^+$ T cell help. When PCA was carried out on helped and unhelped effector CTLs either before (*SI Appendix*, Fig. S3*A*) or after (*SI Appendix*, Fig. S3*A*, *Right*) after peptide stimulation, this significant overlap was even more apparent. While 4,013 DEGs were common to both helped and unhelped effector CTLs, we were able to identify 693 and 279 DEGs unique to unhelped and helped effector CTLs, respectively (*SI Appendix*, Fig. S3*B* and Dataset S1). Gene ontology (GO) analysis of DEGs uniquely found in unhelped effectors identified enrichment for biological processes associated with increased levels of cellular activation, such as cell migration, cell division, microtubule assembly, regulation of GTPase activity, positive regulation of the MAPK cascade, and autophagy (*SI Appendix*, Fig. S3*C*). Moreover, genes associated with immune lineage functions, particularly the positive regulation of genes associated with cytokine production and secretion (IL-12, IL-8, and IL-10 pathways), were also enriched. For those DEGs uniquely identified in helped effector CTLs, GO analysis demonstrated enrichment for genes associated with regulating protein localization, DNA templated transcription, cell projection organization, protein catabolic process, apoptotic process, and adaptive immune response (*SI Appendix*, Fig. S3*D*). Interestingly SATB1, shown to be important for restraining T cell exhaustion (20), was uniquely expressed in helped effector CTLs (Dataset S1). Despite no measurable difference in response magnitude or function, transcriptional profiling indicated that intrinsic molecular profiles distinguished helped from unhelped IAV-specific effector CTL.

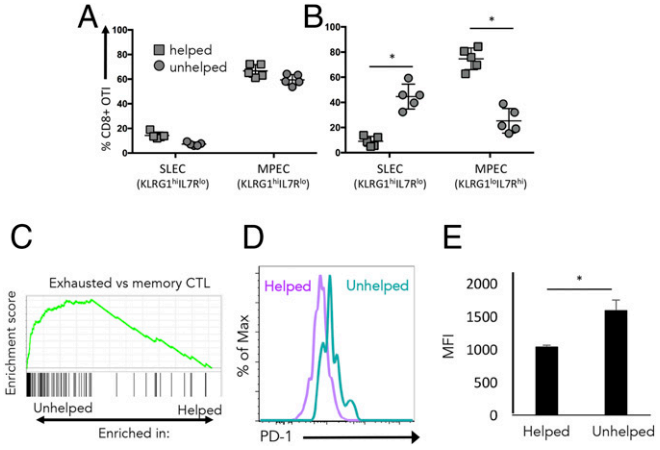

Fig. 3. Phenotypic and transcriptional differences between helped and unhelped virus-specific memory CTL. (*A* and *B*) Congenic (CD45.1), naïve (CD44^{lo}CD62L^{hi}) OT-I T cells (10^4) were adoptively transferred into either uninfected B6 (■) or GK1.5 transgenic mice (unhelped, ●) followed by intranasal infection with 10^4 pfu of A/HKx31-OVA (18). Splenocytes were isolated and stained for CD45.1 and CD8 to detect the proportion of OT-I T cells at either day 8 (effector, *A*), or 28 d after infection (memory, *B*). The proportion of SLEC (KLRG1^{hi}IL-7R^{lo}) or MPEC (KLRG^{lo}IL-7R^{hi}) CTL populations was determined. Data shown is the mean ± SD and are representative of three independent repeats. Significance tested with one-way ANOVA test with Tukey posttest (*$P < 0.001$). (*C*) Gene set enrichment analysis was used to identify enrichment of genes uniquely transcribed in either helped vs. unhelped with gene sets identified from T cell exhaustion found enriched in unhelped memory OT-Is. (*D* and *E*) Unhelped memory OT-Is exhibit higher levels of PD-1 expression. Helped and unhelped memory OT-I CTL were established as described above. PD-1 expression was assessed on splenic memory OT-Is (day 28) by flow cytometry. Shown is a representative histogram for helped (purple line) or unhelped (cyan line) memory OT-Is. (*E*) Quantitation of mean fluorescence intensity as a measure of cell surface levels of PD-1. Data shown mean ± SD and are representative of two independent repeats. Significance tested with one-way ANOVA test with Tukey posttest (*$P < 0.01$).

We then compared the global transcriptional profile of helped and unhelped day 28 memory OT-I CTLs. PCA demonstrated there were major differences in transcriptional signatures between helped and unhelped memory CTLs both before and after peptide stimulation (*SI Appendix*, S4*A*). We identified 261 and 141 DEGs uniquely transcribed in helped vs. unhelped memory CTLs in the resting state, or after peptide stimulation, respectively (Dataset S2). Similar to the analysis of unhelped effector CTLs, GO analysis identified enrichment for genes in unhelped memory IAV-specific CTLs associated with cell cycle, cell division, G2/M transition, negative regulation of cytokines, and regulation of inflammatory responses (*SI Appendix*, S4*B*). Interestingly, several of the genes associated with up-regulation in unhelped memory CTLs included the T cell effector and chemokine receptor genes (*Gzma*, *Gzmb*, *Ccl9*, *Ccr4*, *Ccr6*, *Cx3cr1*) and inhibitory receptors (*Tigit*, *Lag3*, *Klrg1*, *Klra3*, *Itgam*) that have previously associated with T cell exhaustion (*SI Appendix*, Fig. S4*B*) (21). To validate these initial findings, programmed cell death protein 1 (PD-1) expression was examined by flow cytometry on helped and unhelped memory OT-I CTL established after IAV infection. A higher level of cell surface PD-1 expression was observed on unhelped compared with helped memory CTLs (Fig. 3 *D* and *E*). Taken together, these data suggest that CD4 T cell help at the time of IAV-specific CTL priming helps limit T cell exhaustion signatures.

Unhelped vs. Helped Memory CTLs Show Differences in Metabolic Capacity. Differentiation of virus-specific T cells from the naïve to the effector state requires a change in the metabolic pathways utilized for energy production (22). While naïve T cells predominantly utilize oxidative phosphorylation (OXPHOS), effector CTLs up-regulate aerobic glycolysis, an anabolic metabolic pathway that relies on glucose as substrate and supports generation of biomass and nucleotide synthesis for cell division. With transition into memory, OXPHOS again become the predominant pathway but memory CTL maintain a higher mitochondrial load per cell, which manifests as a higher spare respiratory capacity (SRC). It is thought that increased SRC in memory CTL facilitates their long-term survival and their ability to rapidly reengage bioenergetics pathways to sustain a recall response (23).

Rapamycin inhibits the mammalian target of rapamycin (mTOR) pathway, an important environmental sensor that coordinates multiple metabolic pathways (24), and rapamycin treatment has been shown to improve generation of memory cell populations by promoting reversion to OXPHOS. In addition, genes associated with activity of NADH dehydrogenase, which forms complex I in the electron transport chain (ETC) and is an essential component for OXPHOS, were enriched in helped but not unhelped memory CTL (Fig. 4 *A* and *B*). Hence, these data reinforce that unhelped compared with helped IAV-specific memory CTL exhibit an exhausted profile and suggest that unhelped memory CTL exhibit disruption of both mTOR- and OXPHOS-related pathways.

To directly test whether unhelped memory CTLs exhibited defective OXPHOS, we assessed oxygen consumption rates (OCR) using a Seahorse MitoStress Test. This approach can define distinct measures of mitochondrial respiration and OXPHOS (25), including: (*i*) the basal respiration; (*ii*) ATP-coupled respiration via addition oligomycin, which inhibits ATP Synthase; (*iii*) maximal respiration via addition of carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) to uncouple the ETC; and (*iv*) nonmitochondrial respiration via addition of antimycin A and rotenone (Ant/Rot) to inhibit complexes I and II of the ETC. In addition, the difference between basal and maximal OCR is a measure of SRC, which is increased in memory CTLs. We assessed OCR for both the endogenous IAV-specific (D^bNP_{366} and D^bPA_{224}) (Fig. 4 *C* and *D*) and the transferred memory OT-I CTL memory CTLs directly ex vivo (Fig. 4 *E* and *F*). Helped and unhelped memory CTLs had comparable levels of basal respiration, ATP-coupled respiration, nonmitochondrial respiration, and proton leak (Fig. 4 *C*–*F*), but the maximal respiration rate and the SRC was significantly diminished for the unhelped memory CTLs compared with helped memory CTLs (Fig. 4 *C*–*F*). This trend was similar for both endogenous IAV-specific memory CTLs and transferred CD45.1$^+$ OT-I CTLs (Fig. 4 *C*–*F*). Given that SRC has been correlated with the capacity of memory CTL to survive long term and with the capacity of memory CTL to rapidly reengage metabolic pathways upon reactivation, these data suggest that the reduced number of unhelped CTLs at memory timepoints and their defective recall responses may be due to a defective reversion to OXPHOS.

Recent data has also suggested that exhausted T cells have intrinsic defects in the ability to engage glycolysis upon activation (26), and are thereby unable to provide the necessary energy to sustain an optimal recall response. To examine whether unhelped memory CTLs exhibit a similar defect, we measured uptake of a fluorescent glucose analog, 2-(*N*-7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-2-Deoxyglucose (2-NBDG), by endogenous IAV-specific memory CTLs after in vitro peptide stimulation (Fig. 4 *G* and *H*). While both helped and unhelped memory IAV-specific CTLs exhibited glucose uptake after reactivation, helped memory OT-I CTLs exhibited significantly greater glucose uptake compared with unhelped memory IAV-specific CTLs at 5 and 12 h after stimulation (Fig. 4 *G* and *H*). This highlights that unhelped memory CTLs are defective in their ability to reengage glycolysis after activation, consistent with their exhausted phenotype. To assess whether the defect in SRC was due to diminished mitochondrial mass, we assessed stained helped and unhelped OT-Is using MitoTracker Green (*SI Appendix*, Fig. S5). Interestingly, while there was no significant difference, helped OT-Is exhibited a small trend toward higher mitochondrial mass OT-I, as indicated in a shift in peaks. While not conclusive, it suggests that the defect in SRC is due to diminished mitochondrial formation. Overall, our data suggest that the physiological defect in recall capacity exhibited unhelped memory CTLs may be a consequence dysfunctional metabolic processes. Specifically, it seems that unhelped memory CTLs are unable to maintain higher SRC to support survival and facilitate activation and are unable to fully engage the glycolytic pathway once activated to support cell growth and division.

Discussion

We established that the priming of adoptively transferred OT-I CTLs within CD4-deficient hosts resulted in diminished recall responses, supporting previous studies establishing a CD4$^+$ T help requirement for the establishment of endogenous IAV-specific CTL memory. Similar protocols have been used to reach much the same conclusions for the lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes* infection models CTL (19, 26). For example, when LCMV-specific effector CTLs were adoptively transferred into either CD4-competent or CD4-deficient (MHC class II KO) recipients, the contraction of LCMV-specific memory CTL numbers and loss of memory potential was greater in the CD4$^+$ T cell-deficient environment (26). Similarly, we found that OT-I memory T cell numbers were lower and exhibited a more differentiated phenotype (CD62L^{lo} KLRG1^{hi}) in CD4$^+$ T cell-depleted, IAV-OVA–infected mice. It should be noted that the proportion of T cell central memory (T_{CM}) vs. T cell effector memory (T_{EM}) in a memory population can be influenced by both starting precursor frequency and route of infection (27, 28). Hence, the fact that we utilized a protocol involving an intranasal prime/challenge replicating what is seen with human IAV infection may have influenced the of T_{CM}:T_{EM} ratio. While an observed decrease in the number T_{CM} precursors could potentially explain the diminished-memory OT-I recall response in the absence of concurrent help, we have previously shown that KLRG1 and CD62L are poor predictors of memory

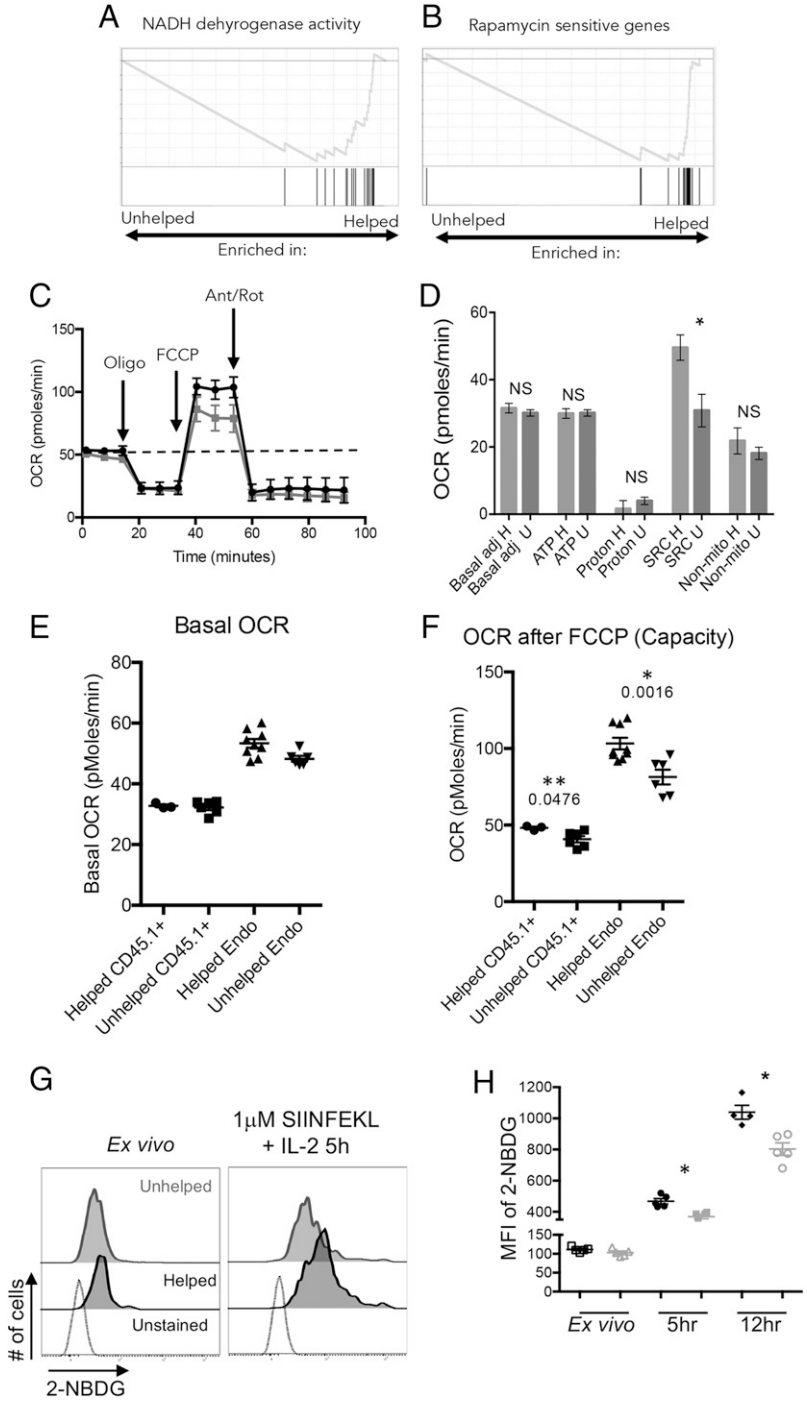

Fig. 4. Unhelped memory CTL exhibit dysfunctional metabolic capacity. (*A* and *B*) Gene set enrichment of RNA-seq data showed unhelped memory OT-Is exhibited lack of genes associated with NADH dehydrogenase activity (*A*) and rapamycin-sensitive pathways (*B*). Positive enrichment indicates enrichment in unhelped memory CTL, negative enrichment indicates enrichment in helped memory CTL. (*C–F*) Mitochondrial respiratory capacity was measured using the MitoStress Test using the Seahorse bioanalyser for both adoptively transferred OT-I and endogenous IAV-specific (D^bNP_{366} and D^bPA_{224}) memory CTL. (*C*) Endogenous memory CTL were pooled from either wild-type (helped, black lines) and GK1.5Tg (unhelped, gray) day 28 after primary A/HKx31-OVA infection. The OCR was measured before induction of metabolic stress with injection of Oligomycin (Oligo) (first arrow). Mitochondrial capacity was tested by injection of the mitochondrial decoupler, FCCP (second arrow). Nonmitochondrial respiration was measured after addition of Ant/Rot to shut down the electron transport chain (third arrow). The dashed line shows the basal OCR with SRC capacity the difference between basal OCR and maximal OCR after FCCP addition. (*D*) Distinct aspects of mitochondrial respiration were determined from analysis of the Seahorse MitoStress Test for $CD8^+$ IAV-specific memory T cells isolated from wild-type (helped, dark gray bars) or GK1.5Tg (unhelped, light gray bars) mice. Significance tested with one-way ANOVA test with Tukey posttest (*$P < 0.0016$). (*E* and *F*) Basal OCR and SRC (OCR after addition of FCCP) is shown for both endogenous and OT-I helped and unhelped memory CTL. Data shown is the mean ± SD and are representative of two independent repeats. Significance tested with one-way ANOVA test with Tukey posttest (*$P < 0.0016$; **$P < 0.0476$). (*G* and *H*) Memory OT-I CTL were generated in WT or GK1.5Tg mice as described above. (*G*) Glucose uptake was measured by culturing unhelped (light gray) or helped (dark gray) memory OT-I CTL in the presence of the glucose analog 2-NBDG, either directly ex vivo or after 5 h of peptide stimulation. (*H*) The mean fluorescence intensity was measured to determine the degree of 2-NBDG uptake for unhelped (black symbols) or helped (gray symbols) either directly ex vivo, or 5 and 12 h after in vitro peptide stimulation. Data shown is the mean ± SD and are representative of two independent repeats. Significance tested with one-way ANOVA test with Tukey posttest (*$P < 0.01$; NS, not significant).

CTL potential for IAV-primed mice (29). Importantly, analysis of the fold-expansion of recalled memory CTLs indicated that the unhelped memory CTLs proliferated less than the comparable helped sets. This suggested that unhelped memory CTLs have an intrinsic defect that limits their capacity to respond following secondary challenge.

The concept that $CD4^+$ T cell help is only required for the maintenance of memory populations is in contrast to other evidence that such help at the time of initial priming is critical for programming $CD8^+$ T cell memory capacity (3, 30). In our study, adoptive transfer of unhelped memory CTLs into $CD4^+$ T cell-competent animals did not restore recall capacity compared with the response profiles for an equal number of helped memory CTLs. Moreover, following IAV challenge, helped memory CTLs were able to respond equivalently in both CD4-competent and -deficient environments when infected soon after transfer. Hence, it would appear that, in the context of IAV infection, $CD4^+$ T cell help is required at the time of initial priming for the maintenance and recall of effective $CD8^+$ T cell memory.

A possible explanation for the need to provide $CD4^+$ T cell help at the time of priming is to ensure that memory CTLs are capable of responding to homeostatic signals, like IL-7 and IL-15. This is supported by previous reports showing that unhelped memory CTLs exhibit lower levels of CD122 (IL-2Rβ), a key coreceptor for IL-15 signaling (31). Otherwise, just how $CD4^+$ T cells impart the "programming signal" to promote memory establishment remains unclear. Some studies suggest that the impact of $CD4^+$ T cell help is indirect. For example, CD40L/CD40-mediated licensing of DCs is required to support mature $CD8^+$ T cell activation and autocrine IL-2 production (30). Another proposed mechanism is that $CD4^+$ T regulatory (Treg) modulation of DC function is required for maturation of high-avidity CTL responses and the establishment of effective CTL memory via ensuring responding CTL are resistant to Treg-mediated supression (9). In this case, the depletion of Tregs before immunization resulted in the overproduction of inflammatory chemokines that, in turn, led to a requirement for in prolonged antigen activation with a subsequent overproliferation of low-avidity CTLs (32). Additionally, a more recent study suggested that Treg-dependent production of IL-10 serves to protect developing memory CTL from inflammatory signals associated with infection, thereby promoting memory CTL formation (33). Thus, it is possible that the absence of Tregs, rather than the lack of specific signals provided by conventional $CD4^+$ T helpers in the GK1.5Tg mice, could be responsible for the diminished recall capacity of the unhelped OT-I memory CTLs. In further studies, it may be of interest to examine whether specific Treg depletion also results in memory dysfunction following acute IAV infection but, as yet, it remains unclear how $CD4^+$ T cells impart the programming signal to promote memory establishment.

We observed that unhelped memory CTLs exhibited phenotypic and transcriptional signatures characteristic of T cell exhaustion (21, 34). Specifically, we observed up-regulation of the inhibitory receptors *Klrg1*, *Klra3*, *Itgam*, *Havcr2*, *Lag3*, and *Tigit*, as well as *Prdm1*, a key regulator of CTL exhaustion (35). Transient $CD4^+$ T cell depletion during the initial stages of chronic LCMV infection results in rapid establishment of severely exhausted $CD8^+$ T cells in persistently infected mice (36). More recently, it has been reported that dysfunctional tumor-specific $CD8^+$ T cell memory occurs in the absence of $CD4^+$ T cell help and is a consequence of prolonged, high antigen load driving effector CTL exhaustion (37). While the primary IAV-specific CTL responses is largely intact in the GK1.5Tg mice, the lack of $CD4^+$ T cells does result in prolonged effector CTL expansion and delayed viral clearance (1). Hence, unhelped effector and memory IAV-specific CTLs may be exposed to factors that promote eventual T cell exhaustion. Interestingly, while we identified "exhaustion signatures," we observed that unhelped memory CTL exhibited similar cytokine production to helped memory CTL upon restimulation. A possible explanation is that unhelped memory establishment is not a true "exhaustion state," and that perhaps installation of effector function is independent of memory establishment. Conversely, this might also reflect that a proportion of naive CTL do not rely on CD4 T cell help for memory establishment or function. A subset of naive T cells, referred to as virtual memory T cells (T_{VM}), express T cell memory markers and exhibit more rapid effector function upon activation (38). One hypothesis is that perhaps functional CTL found in unhelped memory populations may reflect the T_{VM} found within a naive $CD8^+$ T cell population do not require CD4 T cell help to establish memory upon activation.

Following T cell activation, cells shift from predominantly utilizing mitochondrial OXPHOS to aerobic glycolysis, which relies on uptake of glucose to fuel the energy needs of the dividing cell (22), with transition back to a memory associated to a shift back to predominantly OXPHOS. Earlier observations demonstrated that exhausted LCMV-specific CTLs exhibited diminished metabolic capacity with lower levels of glycolytic and mitochondrial respiratory capacity (26). We too were able to demonstrate that the unhelped memory OT-I CTLs exhibited decreased mitochondrial respiratory capacity directly ex vivo and lower levels of glucose uptake after in vitro reactivation, compared with helped memory OT-I CTLs. Of note, concurrent $CD4^+$ T cell involvement does not appear mandatory for reactivation, but we have not assessed whether it is required for reengagement of aerobic glycolysis by memory CTLs. However, we also propose that $CD4^+$ T cell help is required at the time of priming to help ensure that memory CTLs remodel their basal metabolic capacity to support their long-term maintenance and reprogram their metabolic potential to ensure that they respond effectively upon reactivation.

What remains to be determined with regard to the establishment of functional IAV-specific $CD8^+$ T cell memory is whether $CD4^+$ T cell-generated signals acting directly on the responding CTLs install an "optimal" and enduring molecular program or, alternatively, whether this is an intrinsic program that is compromised when activated CTLs undergo a process of "unprotected" (by T help) differentiation, leading to a substantially exhausted phenotype. Certainly, activation of CTLs using allogeneic tumors demonstrated that memory potential was retained if "unhelped" CTL were isolated at earlier phases of the effector response (37). The fact that unhelped OT-I memory CTLs induced after IAV-OVA infection were unable to recover function when transferred into a CD4 competent host indeed suggests that memory potential is irreversibly compromised relatively early after initial antigen exposure. Changes in IAV-specific T cell function are associated with remodeling of the genomic landscape that reflects differentiation state (39–41). Given recent data showing that T cell exhaustion is associated with an altered chromatin landscape compared with bonafide memory T cells (42, 43), it will be of particular interest to examine whether the chromatin landscape of unhelped memory CTL is similar to that observed in exhausted T cells.

While we favor a model whereby $CD4^+$ T help is important for retaining this intrinsic memory potential via limiting extensive $CD8^+$ T cell differentiation, further analysis is required to delineate the precise time after activation when such potential is lost. Moreover, it is unclear whether the defect can be reversed by reconstituting memory potential via drug treatment to restore metabolic function, and thus a capacity for effective recall. While there are some indications this might be possible, further work is required to assess whether such interventions will be more broadly applicable. Any protocol that might allow effector potential to be reactivated in persistent oncogenic or infectious processes obviously merits further investigation.

Materials and Methods

Mice, Viruses, and Infection. Female Ly5.2$^+$ C57BL/6J (B6), Ly5.2 GK1.5Tg, and congenic Ly5.1$^+$ OT-I mice were bred and housed under specific pathogen-free

conditions at either The Peter Doherty Institute for Infection and Immunity animal facility at the University of Melbourne, or the Animal Resource Laboratory at Monash University. For infection, mice were anesthetized and infected intranasally with 10^4 pfu of recombinant A/HKx31 virus engineered to express the $OVA_{257–264}$ peptide (HKx31-OVA) in the neuraminidase stalk (18). For secondary challenge, mice were first primed with 10^7 pfu A/PR8-OVA via intraperitoneal injection, followed by intranasal infection with 10^4 pfu A/HKx31-OVA, at least 28 d after initial priming. All experiments were conducted according to approval obtained from the institutional animal ethics committee at both the University of Melbourne and Monash University.

Adoptive Transfer, Tissue Sampling, Cell Culture, and Flow Cytometry. For adoptive transfers, 10^4 naïve ($CD44^{lo}CD62L^{hi}$) isolated from lymph nodes or 10^3 memory OT-I cells isolated from spleens were injected intravenously 24 h before infection with A/HKx31-OVA intransally into either B6 (helped) or GK1.5Tg (unhelped) mice. Effector and memory $CD8^+$ $CD45.1^+$ OT-I cells (10 and >28 d postinfection, respectively) were generated from lymphocyte preparations (10^7/mL) resuspended in PBS/0.1% FCS and stained with monoclonal antibodies for anti–CD8a- FITC (clone 53–6.7; eBiosciences), and anti-CD45.1-allophycocyanin (APC; clone A20; eBiosciences) to detect OT-I cells. Naive cells ($CD44^{lo}CD8^+$) were stained with CD44-FITC (clone IM7; eBiosciences) and CD8-APC. For secondary challenge, memory mice were infected with 10^2 pfu A/PR8-OVA intranasally at least 28 d after initial priming. Cells were sorted with a FACS Aria (BD Biosciences). Samples were then analyzed with a FACS Canto (BD Biosciences) and FlowJo software (Tree Star). Intracellular cytokine staining was performed by incubation of cells with 1 μg/mL of $OVA_{257–264}$ peptide (amino acid sequence, SIINFEKL) for 5 h in the presence of IL-2 and Brefeldin A at 37 °C, 5% CO_2 before surface staining with either anti–CD8-PerCPCy5.5 and CD45.1-Pacific Blue. Cells were then fixed, permeabilized, and intracellularly stained with IFN-γ–FITC (clone XMG1.2; eBiosciences), TNF-α–APC (clone MP6-XT22; eBiosciences) and IL-2–PE (clone JES6-5H4; eBiosciences). Data were acquired on a LSRFortessa with FACSDiva software (BD Immunocytometry Systems). Postacquisition data analyses were performed using FlowJo software (Tree Star).

Analysis of the IAV-Specific $CD8^+$ T Cell Response. Splenocytes and cells from bronchoalveolar lavage (BAL) were stained with either CD45.1-PE and CD8-APC, or CD8-APC and D^bNP_{366}-PE (44) and D^bPA_{224}-PE tetramers (45), at 4 °C for 30 min and data acquired by FACSCantoII.

RNA Sequencing. RNA sequencing was carried out according to Russ et al. (40). Briefly, RNA was extracted from sorted memory OT-I CTL and extracted from TRIzol suspensions using the Zymo Research Direct-zol RNA miniprep kit. RNA-seq libraries were prepared used Illumina's TruSeq RNA v2 sample preparation protocol according to the manufacturer's instructions. The cDNA libraries were run on an Illumina HiSeq2000. Data quality was checked with fastqc software. Paired-end RNA-seq data were aligned to mouse mm10 genome using TopHat (with Bowtie2). Only concordant pairs with mapping quality >10 were kept. The number of reads assigned to each gene was found using Bioconductor R package. Count data were analyzed using edgeR Bioconductor package (GLM formulation). Before doing this, genes that did not have ≥3 counts in every sample for at least one group were filtered out. Genes were declared differentially expressed if they had a false-discovery rate <0.05 and $\log_2$ fold-change >1. PCA, implemented in EdgeR function of plotMDS (in two dimensions), was used for data visualization. GO analysis was carried out using DAVID (46, 47). Gene set enrichment analysis was carried out using the SeqGSEA Bioconductor package, v3.6 (48).

Seahorse Analysis. Sorted, antigen-specific $CD8^+$ memory cells from wild-type mice (helped) or GK1.5Tg mice (unhelped) were plated at 2×10^5 in XF cell culture microplates coated with CellTak (Corning). Cell respiration was tracked using the MitoStress Test, as per the manufacturer's instructions on a Seahorse XFe96 Bioanalyser. OCR was assessed over time with addition of 1 μM oligomycin, 1.2 μM FCCP, and finally 500 nM rotenone/antimycin A, with OCR readings taken for 3-min intervals with 3 min of mixing after each addition. Data generated was analyzed using PRISM.

ACKNOWLEDGMENTS. This work was supported by National Health and Medical Research Council of Australia Program Grant 5671222 (to S.J.T. and P.C.D.) and Project Grant APP1003131 (to S.J.T.). N.L.L.G. is supported by an Australian Research Council Future Fellowship. S.J.T. is supported by a National Health and Medical Research Council Principal Research fellowship.

1. Belz GT, Wodarz D, Diaz G, Nowak MA, Doherty PC (2002) Compromised influenza virus-specific CD8(+)-T-cell memory in CD4(+)-T-cell-deficient mice. *J Virol* 76: 12388–12393.
2. Janssen EM, et al. (2003) CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 421:852–856.
3. Shedlock DJ, Shen H (2003) Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* 300:337–339.
4. Sun JC, Bevan MJ (2003) Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* 300:339–342.
5. Bennett SR, et al. (1998) Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393:478–480.
6. Rapetti L, Meunier S, Pontoux C, Tanchot C (2008) CD4 help regulates expression of crucial genes involved in CD8 T cell memory and sensitivity to regulatory elements. *J Immunol* 181:299–308.
7. Williams MA, Tyznik AJ, Bevan MJ (2006) Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature* 441:890–893.
8. Ballesteros-Tato A, León B, Lee BO, Lund FE, Randall TD (2014) Epitope-specific regulation of memory programming by differential duration of antigen presentation to influenza-specific CD8(+) T cells. *Immunity* 41:127–140.
9. Ballesteros-Tato A, León B, Lund FE, Randall TD (2013) CD4+ T helper cells use CD154-CD40 interactions to counteract T reg cell-mediated suppression of CD8+ T cell responses to influenza. *J Exp Med* 210:1591–1601.
10. Janssen EM, et al. (2005) CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature* 434:88–93.
11. Marzo AL, et al. (2004) Fully functional memory CD8 T cells in the absence of CD4 T cells. *J Immunol* 173:969–975.
12. Fang M, Remakus S, Roscoe F, Ma X, Sigal LJ (2015) CD4+ T cell help is dispensable for protective CD8+ T cell memory against mousepox virus following vaccinia virus immunization. *J Virol* 89:776–783.
13. Ramsburg EA, Publicover JM, Coppock D, Rose JK (2007) Requirement for CD4 T cell help in maintenance of memory CD8 T cell responses is epitope dependent. *J Immunol* 178:6350–6358.
14. Tripp RA, Sarawar SR, Doherty PC (1995) Characteristics of the influenza virus-specific CD8+ T cell response in mice homozygous for disruption of the H-2IAb gene. *J Immunol* 155:2955–2959.
15. Olson MR, et al. (2014) CD154+ CD4+ T-cell dependence for effective memory influenza virus-specific CD8+ T-cell responses. *Immunol Cell Biol* 92:605–611.
16. Jenkins MR, et al. (2008) Cell cycle-related acquisition of cytotoxic mediators defines the progressive differentiation to effector status for virus-specific CD8+ T cells. *J Immunol* 181:3818–3822.
17. Zhan Y, Martin RM, Sutherland RM, Brady JL, Lew AM (2000) Local production of anti-CD4 antibody by transgenic allogeneic grafts affords partial protection. *Transplantation* 70:947–954.
18. Jenkins MR, Webby R, Doherty PC, Turner SJ (2006) Addition of a prominent epitope affects influenza A virus-specific CD8+ T cell immunodominance hierarchies when antigen is limiting. *J Immunol* 177:2917–2925.
19. Sun JC, Williams MA, Bevan MJ (2004) CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat Immunol* 5:927–933.
20. Stephen TL, et al. (2017) SATB1 expression governs epigenetic repression of PD-1 in tumor-reactive T cells. *Immunity* 46:51–64.
21. Wherry EJ, et al. (2007) Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27:670–684.
22. Chang CH, et al. (2013) Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* 153:1239–1251.
23. van der Windt GJ, et al. (2012) Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development. *Immunity* 36:68–78.
24. Powell JD, Delgoffe GM (2010) The mammalian target of rapamycin: Linking T cell differentiation, function, and metabolism. *Immunity* 33:301–311.
25. van der Windt GJ, Chang CH, Pearce EL (2016) Measuring bioenergetics in T cells using a seahorse extracellular flux analyzer. *Curr Protoc Immunol* 113:3.16B.1–3.16B.14.
26. Bengsch B, et al. (2016) Bioenergetic insufficiencies due to metabolic alterations regulated by the inhibitory receptor PD-1 are an early driver of CD8(+) T cell exhaustion. *Immunity* 45:358–373.
27. Denton AE, et al. (2011) Affinity thresholds for naive CD8+ CTL activation by peptides and engineered influenza A viruses. *J Immunol* 187:5733–5744.
28. Klonowski KD, et al. (2006) CD8 T cell recall responses are regulated by the tissue tropism of the memory cell and pathogen. *J Immunol* 177:6738–6746.
29. Croom HA, et al. (2011) Memory precursor phenotype of CD8+ T cells reflects early antigenic experience rather than memory numbers in a model of localized acute influenza infection. *Eur J Immunol* 41:682–693.
30. Feau S, Arens R, Togher S, Schoenberger SP (2011) Autocrine IL-2 is required for secondary population expansion of CD8(+) memory T cells. *Nat Immunol* 12:908–913.
31. Khanolkar A, Fuller MJ, Zajac AJ (2004) CD4 T cell-dependent CD8 T cell maturation. *J Immunol* 172:2834–2844.
32. Pace L, et al. (2012) Regulatory T cells increase the avidity of primary CD8+ T cell responses and promote memory. *Science* 338:532–536.
33. Laidlaw BJ, et al. (2015) Production of IL-10 by CD4(+) regulatory T cells during the resolution of infection promotes the maturation of memory CD8(+) T cells. *Nat Immunol* 16:871–879.
34. Provine NM, et al. (2016) Immediate dysfunction of vaccine-elicited CD8+ T cells primed in the absence of CD4+ T cells. *J Immunol* 197:1809–1822.

35. Shin H, et al. (2009) A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. *Immunity* 31:309–320.
36. Matloubian M, Concepcion RJ, Ahmed R (1994) CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J Virol* 68:8056–8063.
37. Kim J, et al. (2015) Memory programming in CD8(+) T-cell differentiation is intrinsic and is not determined by CD4 help. *Nat Commun* 6:7994.
38. Haluszczak C, et al. (2009) The antigen-specific CD8+ T cell repertoire in unimmunized mice includes memory phenotype cells bearing markers of homeostatic expansion. *J Exp Med* 206:435–448.
39. Denton AE, Russ BE, Doherty PC, Rao S, Turner SJ (2011) Differentiation-dependent functional and epigenetic landscapes for cytokine genes in virus-specific CD8+ T cells. *Proc Natl Acad Sci USA* 108:15306–15311.
40. Russ BE, et al. (2014) Distinct epigenetic signatures delineate transcriptional programs during virus-specific CD8(+) T cell differentiation. *Immunity* 41:853–865.
41. Russ BE, et al. (2017) Regulation of H3K4me3 at transcriptional enhancers characterizes acquisition of virus-specific $CD8^+$ T cell-lineage-specific function. *Cell Rep* 21: 3624–3636.
42. Pauken KE, et al. (2016) Epigenetic stability of exhausted T cells limits durability of reinvigoration by PD-1 blockade. *Science* 354:1160–1165.
43. Sen DR, et al. (2016) The epigenetic landscape of T cell exhaustion. *Science* 354: 1165–1169.
44. Flynn KJ, et al. (1998) Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. *Immunity* 8:683–691.
45. Belz GT, Xie W, Altman JD, Doherty PC (2000) A previously unrecognized H-2D(b)-restricted peptide prominent in the primary influenza A virus-specific CD8(+) T-cell response is much less apparent following secondary challenge. *J Virol* 74:3486–3493.
46. Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4:44–57.
47. Huang da W, Sherman BT, Lempicki RA (2009) Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 37: 1–13.
48. Wang X, Cairns MJ (2014) SeqGSEA: A bioconductor package for gene set enrichment analysis of RNA-seq data integrating differential expression and splicing. *Bioinformatics* 30:1777–1779.