Walking the wire

Post-transcriptional regulation of T cell effector functions in health and disease

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Chapter 4

TLR-mediated innate production of IFN-γ by CD8\(^+\) T cells is independent of glycolysis

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Abstract
CD8+ T cells can respond to unrelated infections in an antigen-independent manner. This rapid innate-like immune response allows antigen-experienced T cells to alert other immune cell types to pathogenic intruders. Here, we show that murine CD8+ T cells can sense toll-like receptor (TLR) 2 and TLR7 ligands, resulting in rapid production of IFN-γ, but not of TNF-α and IL-2. Importantly, antigen-experienced T cells activated by TLR ligands produce sufficient IFN-γ to augment the activation of macrophages.

In contrast to antigen-specific reactivation, TLR-dependent production of IFN-γ by CD8+ T cells relies exclusively on newly synthesized transcripts without inducing mRNA stability. Furthermore, transcription of IFN-γ upon TLR triggering depends on the activation of phosphatidylinositol 3-kinase (PI3K) and serine-threonine kinase Akt, and protein synthesis relies on the activation of the mechanistic target of rapamycin (mTOR). We next investigated which energy source drives the TLR-induced production of IFN-γ. While antigen-specific cytokine production requires a glycolytic switch for optimal cytokine release, glucose availability does not alter the rate of IFN-γ production upon TLR-mediated activation. Rather, mitochondrial respiration provides sufficient energy for TLR-induced IFN-γ production.

To our knowledge this is the first report describing that TLR-mediated bystander activation elicits a helper phenotype of CD8+ T cells. It induces a short boost of IFN-γ production that leads to a significant but limited activation of antigen-experienced CD8+ T cells. This activation suffices to prime macrophages, but keeps T cell responses limited to unrelated infections.
Introduction

CD8⁺ T cells are critical members for defense against intracellular pathogens. They rapidly shift from a quiescent to a highly active state upon reinfection with the pathogen, which allows them to produce massive amounts of effector molecules within a few hours. IFN-γ is a central effector molecule of CD8⁺ T cells that has a broad spectrum of activity. IFN-γ not only dampens the growth of pathogens, it also recruits neutrophils to the site of infection and activates immune cells such as macrophages to potentiate the innate immune response. In humans, intact IFN-γ production and IFN-γ receptor signaling is essential for anti-mycobacterial immunity.

T cells reside in lymphoid and in non-lymphoid tissues, such as skin, lung, kidney, and the female reproductive tract. This tissue-specific localization ensures that T cells exert their effector function right at the entry site of the pathogen and thereby limit the spreading of the infection. CD8⁺ T cells are not only important during reinfections with the cognate pathogen. They also have the capacity to respond to unrelated pathogenic intruders. In the absence of antigen recognition through the T cell receptor (TCR), CD8⁺ T cells can acquire innate defense functions. Inflammatory cytokines can directly activate antigen-experienced T cells and, for example, induce the production of IFN-γ or granzyme. The production of IFN-γ during this bystander activation supports the immune response against unrelated infections. To achieve this cross-protection, innate immune cells like DCs and NK cells are recruited to the site of infection, and macrophages and neutrophils become activated by IFN-γ.

While it has been established that antigen-experienced CD8⁺ T cells respond to inflammatory cytokines, it is not known whether also other stimuli can trigger T cells in an antigen-independent manner. For instance, ligands for toll-like receptors (TLRs) signify the presence of microbial non-self and are major activators of innate immunity. We therefore asked whether TLR ligands would also mobilize innate immune functions by previously activated T cells. Earlier studies have shown that bacterial lipoproteins and CpG sequences provide costimulation to the TCR engagement during T cell activation. In particular, costimulation through TLR2 triggering lowers the threshold of antigen required for an optimal activation of CD8⁺ T cells. In addition, TLR2-mediated costimulation promotes the development and maintenance of memory T cells. However, whether TLR ligands alone can drive the activation of antigen-experienced T cells is yet to be determined. In the present study, we show that murine antigen-experienced CD8⁺ T cells can be directly activated by TLR2 and TLR7 ligands, but not by TLR3, TLR4, TLR5, or TLR9 ligands. TLR2- and TLR7-mediated activation of CD8⁺ T cells does not induce production of TNF-α or IL-2, but results in production of IFN-γ that is sufficient to alert and prime macrophages. TLR-driven IFN-γ production is of short nature as it relies on newly synthesized mRNA without increasing the rate of translation by stabilizing Ifng transcripts. Similar to what is found for primary T cell activation and for reactivation of memory T cells with cognate antigen, TLR-mediated generation of Ifng transcripts relies on the PI3K-Akt signaling axis. Protein production is driven by mTOR, a downstream target of PI3K-Akt, for both TLR and TCR mediated activation. When we investigated the source of energy employed during T cell activation, we found different requirements for TLR-mediated and antigen-driven stimulation. While TCR triggering depends on glycolysis to reach optimal cytokine production during recall responses, external sources of glucose are not required for the production of IFN-γ upon TLR.
triggering. Instead, the energy generated by mitochondrial respiration is sufficient to fully support the TLR-dependent innate-like production of IFN-γ. In conclusion, we show that bystander activation of T cells through TLRs leads to a short but biologically significant burst of IFN-γ production, which relies exclusively on newly synthetized transcripts and energy generated by mitochondrial respiration.

**Results**

**CD8+ T cell triggering with TLR2 and TLR7 ligands induces IFN-γ production in an antigen-independent manner**

We first set out to determine whether antigen-experienced T cells can be bystander activated by TLR ligands. We generated antigen-experienced T cells by activating MACS-enriched CD8+ OT-I T cells for 20 hours with MEC.B7.SigOVA cells expressing the cognate antigen OVA257-264 and the costimulatory molecule CD80. This model system results in full T cell activation and allows for subsequent development of bona fide memory T cells in vivo. Upon activation, T cells were allowed to rest in the absence of antigen for several days. Within 24 hours of antigen removal, T cells ceased to produce IFN-γ protein but, when activated again with cognate antigen, they generated substantial amounts of IFN-γ within a few hours (Fig S1A).

In line with previous studies in memory T cells, in vitro generated antigen-experienced CD8+ T cells express transcripts for TLR2, TLR3, TLR5, TLR7, and TLR9 (Fig 1A). Nonetheless, only stimulation with the TLR2 ligand Pam3CysSK4 (Pam3) and the TLR7 ligand R848 led to production of IFN-γ in T cells (Fig 1B). All other TLR ligands tested, i.e. TLR3 ligand poly(I:C), TLR4 ligand LPS, TLR5 ligand flagellin and TLR9 ligands CpGA and CpGB, failed to do so (Fig 1B).

To examine whether the observed TLR-mediated IFN-γ production by CD8+ T cells was a direct effect, or driven indirectly by myeloid cell impurities present after the MACS-purification procedure, we compared the composition of MACS-purified T cells directly after selection, and after 3 days of culture prior to the TLR activation. MACS selection of CD8+ T cells led to a substantial loss of CD11b+ and CD11c+ cells, that was even further decreased when T cells were rested for 3 days, resulting in a T cell purity >99% (Fig 1C-1D). To exclude that TLR-induced IL-12 production by possible contaminations of DCs or macrophages was the driving force for IFN-γ production in T cells, resting T cells were stimulated with Pam3 and R848 in the presence of the IL-12 neutralizing antibody. In this setting, IFN-γ production by T cells was not at all affected (Fig S1B). By using FACS-sorted naive CD44lo CD62Lhi CD8+ OT-I T cells for primary T cell activation, we confirmed that possible contaminations were not the driving force of TLR-dependent IFN-γ production by T cells. This procedure reached a purity >99.9% prior to primary T cell activation (Fig 1E) and, as expected, contamination of myeloid cells was not observed in FACS-sorted T cells (Fig 1E). Importantly, irrespective of the method used for T cell isolation prior to primary T cell activation, i.e. MACS-selected or FACS-sorted cells, we found that TLR2- and TLR7-mediated activation of antigen-experienced CD8+ T cells triggered the production of IFN-γ (Fig 1F-1G).

Therefore, we conclude that TLR triggering acted directly on the T cells.

Interestingly, CD8+ T cells specifically produced IFN-γ upon TLR triggering, while two other critical cytokines for T cell function, TNF-α and IL-2, and the degranulation marker CD107a
were barely detectable (Fig 2A, Fig S1C and data not shown). The TLR-mediated production of IFN-γ was robust over a longer culture period of T cells, from 3 days up to 15 days of rest (Fig 2B, Fig S1D). The response to Pam3 and R848 was dose- and time-dependent, with a maximal IFN-γ production level within the first 6h of stimulation (Fig 2C-2D). Furthermore,
Figure 2: TLR-induced IFN-γ production occurs in an antigen-independent manner

(A) Representative dot plots of IFN-γ and TNF-α (upper panel), and of IFN-γ and IL-2 production (lower panel) of MACS-selected T cells (rested for 3 days) that were stimulated with 5μg/ml Pam3 or 10μg/ml R848 for 6h. (B) Activation of T cells with Pam3 and R848 upon 3 (upper panel) and 15 (lower panel) days of rest. (C) Resting T cells were stimulated with Pam3 or R848 using increasing concentrations (0-10μg/ml) or (D) for indicated time intervals (0-8h) with 1μg/ml Pam3 or 10μg/ml R848. Graphs shows data pooled from 4 independently performed experiments ± SD. (E) IFN-γ production of rested (4 days, (-)) CD8+ (upper panel) and CD4+ (lower panel) T cells from C57BL/6J mice, or after 6h activation with Pam3 or R848. (F) Resting OT-I T cells were pretreated for 30 min with different concentrations of cyclosporine A (CsA) and stimulated for 6h with 100nM OVA257–264, 5μg/ml Pam3, or with 10μg/ml R848. Graphs depict the relative percentage of IFN-γ producing CD8+ T cells compared to T cells reactivated in the absence of CsA (mean ± SD; n=4). (G) T cell activation with Pam3 or R848 in FCS-free medium. (A-B, E) Data are representative of 4 independently performed experiments.
TLR-dependent innate activation is more pronounced in CD8\(^+\) T cells than in CD4\(^+\) T cells. While CD8\(^+\) T cells from C57BL/6 mice clearly responded to stimulation with TLR2 and TLR7 ligands, only a minor fraction of the CD4\(^+\) T cell population produced IFN-\(\gamma\) within 6 hours (Fig 2E).

We next confirmed that the IFN-\(\gamma\) produced by T cells was a direct result of the TLR-triggering and that it was occurring in an antigen-independent fashion. To this end, we activated T cells in the presence of cyclosporine A (CsA) to block TCR-mediated calcineurin activation, and consequently, TCR-mediated IFN-\(\gamma\) production. While CsA effectively inhibited the production of IFN-\(\gamma\) in OVA\(_{257-264}\)-activated OT-I T cells in a dose-dependent manner (Fig 2F), CsA had no effect on the IFN-\(\gamma\) production upon TLR2- or TLR7-triggering (Fig 2 F). This finding reveals that T cells specifically responded to TLR activation and not to possible residual traces of antigen from the primary stimulation. Furthermore, TLR-activated T cells also effectively produced IFN-\(\gamma\) in FCS-free culture medium (Fig 2G), demonstrating that TLR-mediated activation did not rely on additional triggers from FCS-derived cytokines or growth factors. Taken together, we show here that CD8\(^+\) T cells respond directly to pathogen-derived danger signals via TLRs by producing IFN-\(\gamma\).

**TLR-dependent activation of T cells augments macrophage responses**

Previous studies have shown that IFN-\(\gamma\) synergizes with TLR ligands to activate macrophages\(^{22, 23, 34}\). In light of our findings that TLR engagement triggered the production of IFN-\(\gamma\) by antigen-experienced T cells, we examined whether the amount of IFN-\(\gamma\) produced was sufficient to support the activation of macrophages. To this end, we harvested supernatant from resting T cells that were activated for 6 hours with Pam3, which resulted in about 10% IFN-\(\gamma\)-producing T cells (data not shown). When we cultured murine bone marrow-derived macrophages from wild-type mice (WT) for 5 hours with the T cell-derived supernatant, we found a significant induction of \(\text{Tnfa}\) transcripts, a signature for activated macrophages (Fig 3A). Importantly, MyD88\(^{-/-}\) macrophages that completely fail to respond to TLR2 ligands\(^{35}\) also exhibited an increase in \(\text{Tnfa}\) transcripts, albeit to a lesser extent than WT macrophages (Fig 3A). To address whether IFN-\(\gamma\) produced by Pam3-activated T cells was the factor that

![Figure 3: IFN-\(\gamma\) production by TLR2-activated T cells enhances macrophage responses](image-url)

T cells (rested for 6 days) were stimulated for 6h with 5\(\mu\)g/ml Pam3, or left untreated (-) (n=3 mice). BM derived macrophages from (A-C) C57BL/6J WT and MyD88\(^{-/-}\) mice or from (B-D) Balb-c WT and IFN-\(\gamma\)R\(^{-/-}\) mice were cultured for 5h in T cell supernatant produced as described above. (A-B) \(\text{Tnfa}\) and (C-D) \(\text{Il10}\) mRNA expression by macrophages was analyzed by RT-PCR. Graphs shows data pooled from 2 independently performed experiments ± SD. [Paired Student \(t\)-test; \(n=6\) per group; *\(p<0.05\); **\(p<0.01\)].
contributed to the activation of MyD88−/− macrophages, we exposed macrophages from IFN-γR−/− mice to the T cell supernatant. In contrast to direct Pam3 activation of IFN-γR deficient macrophages that induced similar levels of Tnfa transcripts when compared to WT (Fig S2A), Tnfa transcript levels upon exposure to the T cell supernatant were significantly reduced (Fig 3B). These data thus demonstrate that IFN-γ produced by T cells is at least in part responsible for the induction of Tnfa transcripts.

Exposure of macrophages to IFN-γ was shown to suppress the production of the anti-inflammatory cytokine IL-10. MyD88−/− macrophages completely failed to produce Il10 transcripts (Fig 3C), indicating that Il10 transcription is driven by TLR triggering. To determine the role of IFN-γ in the production of IL-10, we measured the responsiveness of IFN-γR−/− macrophages. Interestingly, while Pam3 activation induces equal levels of Il10 transcripts in macrophages from both genetic backgrounds (Fig S2B), the lack of IFN-γR signaling resulted in a 4-fold increase of Il10 transcripts compared to WT cells (Fig 3D), demonstrating that the T cell-derived IFN-γ helps to contain the TLR-mediated induction of IL-10 in macrophages. Together, we show that TLR-dependent production of IFN-γ by CD8+ T cells is sufficient to enhance macrophage responses to TLR ligands. It therefore shows that TLR-driven innate activation of CD8+ T cells is biologically meaningful.

**TLR triggering promotes IFN-γ protein production without stabilizing its mRNA**

We next determined how the production of IFN-γ was governed in antigen-experienced T cells upon TLR triggering. We found that Pam3-mediated activation resulted in substantial IFN-γ protein production (Fig 4A), albeit to a lesser extent than antigen-specific activation of with the cognate peptide (OVA257-264; Fig 4A). We also compared the kinetics of Ifng mRNA in TCR or Pam3-activated T cells. Ifng mRNA in antigen-reactivated T cells peaked 4 hours after activation and levels remained high for a prolonged period (Fig 4B). Induction of Ifng mRNA by Pam3 was weaker and more transient, peaking already within the first 2 hours after stimulation, and rapidly declining at 4 hours post stimulation (Fig 4B). This transient induction upon TLR2 stimulation was also reflected by a short half-life of the Ifng mRNA. When the de novo mRNA synthesis blocking reagent Actinomycin D (ActD) was added 3 hours after Pam3-mediated stimulation, we found that Ifng mRNA was rapidly degraded and that its half-life (t1/2 = 30 min) was comparable to the steady state half-life of Ifng mRNA in unstimulated T cells (resting; Fig 4C). In contrast, OVA257-264-stimulation of resting T cells stabilized Ifng mRNA (t1/2 > 2h; Fig 4C). Interestingly, while peptide stimulation induced Ifng mRNA stabilization already at 1h after T cell activation, TLR triggering failed to do so at any time point measured (Fig 4D-4E). This finding shows that TLR activation is sufficient to promote rapid transcription and protein production, but lacks the signals to stabilize Ifng mRNA that is required for sustained protein production. To further examine whether TLR-dependent production of IFN-γ relied primarily on de novo mRNA transcription, we measured the IFN-γ protein levels of TLR- or TCR-triggered T cells that were activated in the presence of ActD. Blocking transcription with ActD for 1 to 3 hours only affected the IFN-γ protein production upon Pam3 stimulation, but not upon OVA257-264 peptide stimulation (Fig 4F-4G).

Our data thus show that the TLR-dependent production of IFN-γ protein primarily depends on mRNA transcription, and not on mRNA stabilization. This finding suggests that the rapid degradation of newly synthetized Ifng mRNA upon TLR triggering ensures a rapid yet limited production of IFN-γ by T cells in response to unrelated infections.
CD8+ T cells sense TLRs and produce IFN-γ

The PI3K-Akt pathway regulates $\text{Ifng}$ mRNA transcription, and mTOR promotes protein production

TLR2 engagement during a primary activation of CD8+ T cells promotes cell survival and memory T cell formation via the PI3K-Akt pathway. On top of that, activation of mTOR signaling is required to drive the costimulatory effect of TLR2 triggering to increase the production of effector molecules. To investigate whether these signaling pathways are also engaged during TLR-mediated bystander activation of antigen-experienced T cells, we measured the phosphorylation of Akt, mTOR and the ribosomal protein S6K1 (S6), a direct target of mTORC1, by flow cytometry 30 minutes after stimulation with Pam3 or OVA peptide. Both stimuli induced phosphorylation of Akt, mTOR and S6. However, antigen-specific stimulation resulted in more pronounced activation of the PI3K-Akt and mTOR pathway than Pam3 stimulation (Fig 5A). Of note, blocking PI3K with the chemical inhibitor ZTSDK474 (ZTS) prior to T cell activation significantly reduced the staining with antibodies against phosphorylated Akt, mTOR and S6, documenting the specificity of the measured

![Image](image-url)
Figure 5: PI3K-Akt and mTOR pathways regulate IFN-γ production at transcriptional and translational level, respectively.

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CD8⁺ T cells sense TLRs and produce IFN-γ

Figure 5: PI3K-Akt and mTOR pathways regulate IFN-γ production at transcriptional and translational level, respectively.

(A) Akt, mTOR and S6 phosphorylation was analyzed by flow cytometry in resting T cells (-) or 30 minutes after T cell activation with 5μg/ml Pam3 or with 100nM OVA²⁵⁷-²⁶⁴ peptide. (B-E) Resting T cells were pre-incubated for 30 min with 1μg/ml PI-103, 10μM ZTSK474 (ZTS), 30μM IC87114 (IC), 2μg/ml Akti1/2 prior to activation with Pam3 or OVA²⁵⁷-²⁶⁴. (B-C) IFN-γ protein and (D-E) Ifng mRNA levels were determined after 6h and 1h activation, respectively. n=3 mice per group (mean ± SD) [one-way ANOVA with Dunnett’s multiple comparison to untreated control; ****p<0.0001] (D-E) [Paired Student t-test; ****p<0.0001]. (F-G) T cells were pre-incubated for 30 min with indicated concentration of rapamycin or left untreated (-), and the percentage of IFN-γ-producing T cells was determined by intracellular cytokine staining after stimulation with Pam3 or OVA²⁵⁷-²⁶⁴ for 6h. Graphs are representative for (upper panels), or pooled from (lower panels) 4 independently performed experiments ± SD [one-way ANOVA with Dunnett’s multiple comparison to untreated control; *p<0.05; **p<0.005; ***p<0.001; ****p<0.0001]. (H-J) T cells were left untreated (-), or were treated with indicated concentrations of rapamycin prior to 1h of activation to determine Ifng mRNA levels. [n=6 mice (mean ± SD); one-way ANOVA with Dunnett’s multiple comparison to untreated control; *p<0.05]. (I-J) Upon 1h activation with Pam3 or OVA²⁵⁷-²⁶⁴, 10μg/ml Actinomycin D was added to the culture, and T cells were harvested at the indicated time points to determine Ifng mRNA stability.

phosphorylation signal (Fig S3A).

To determine the relative contribution of PI3K-Akt and mTOR signaling in controlling the production of IFN-γ, we pretreated T cells with specific inhibitors prior to activation with Pam3 or OVA²⁵⁷-²⁶⁴ peptide. When we inhibited the PI3K-Akt axis with PI-103 (a multi-targeted PI3K inhibitor), ZTS (a pan3-PI3K inhibitor), IC87114 (IC, a selective inhibitor of p110δ), or with the Akti1/2 inhibitor, IFN-γ production was effectively blocked, both at protein level and mRNA level (Fig 5B-5C, and Fig 5D-5E, respectively), regardless of the stimulus used.

We next examined the role of mTOR in promoting IFN-γ production. While μg ranges of rapamycin (1 to 5μg/ml) were required to limit the production of IFN-γ upon OVA²⁵⁷-²⁶⁴ peptide stimulation, the effect of rapamycin on TLR2 activation was already measurable at ng ranges (10 to 50ng/ml) (Fig 5F-5G). To determine whether the different responsiveness to rapamycin was a result of different stimuli employed, or due to the strength of signal provided, we activated antigen-experienced T cells with low concentrations of OVA²⁵⁷-²⁶⁴ peptide that induced similar levels of IFN-γ as Pam3 stimulation (Fig S3B). Also this suboptimal antigen stimulation required high concentrations of rapamycin to inhibit IFN-γ production, and the phosphorylation levels of Akt, mTOR and S6 were higher when compared to Pam3 stimulation (Fig S3C-3D). Together, these results indicate that rapamycin sensitivity was not merely correlated with the magnitude of IFN-γ production.

Intriguingly, even though rapamycin effectively blocked protein production of IFN-γ upon both TLR and TCR triggering, the initial transcription rate was not affected. Independently of the stimulus, or the concentration of rapamycin used, no differences in Ifng mRNA were observed (Fig 5H). Furthermore, mTOR inhibition with 20ng/ml or 5μg/ml of rapamycin did not change the mRNA half-life of Ifng in T cells activated with Pam3 or OVA²⁵⁷-²⁶⁴ peptide (Fig 5I-5J). Thus, these data imply that mTOR activity in T cells primarily promotes the speed and/or the efficiency of translation.

Taken together, our results show that the PI3K-Akt pathway regulates Ifng mRNA transcription, while mTOR is an important modulator of IFN-γ protein translation in both TCR- and TLR-dependent activation of antigen-experienced T cells. Since reduced mTOR and S6 phosphorylation levels correspond to a lower rate of IFN-γ production upon TLR2 triggering, mTOR may be a limiting factor for protein synthesis.
Both aerobic glycolysis and mitochondrial respiration generate the energy required during T cell activation

T cell activation requires a high production of energy to meet the metabolic demands of full effector functions\textsuperscript{37, 38, 39, 40}. This can be generated through oxidative phosphorylation (OXPHOS) and/or aerobic glycolysis\textsuperscript{41, 42}. Memory T cells have an increased glycolytic capacity compared to naïve T cells\textsuperscript{42, 43}, and the PI3K-Akt and mTORC2 pathway was found to drive the immediate glycolytic switch upon TCR-mediated activation of memory T cells to produce the required energy\textsuperscript{43}. We therefore questioned whether TLR triggering of antigen-experienced T cells also resulted in an immediate switch towards aerobic glycolysis. We measured the extracellular acidification rate (ECAR, an indicator of aerobic glycolysis) and the oxygen consumption rate (OCR, an indicator of OXPHOS) of Pam3 or OVA\textsubscript{257–264} peptide activated T cells with the “in-Seahorse” activation assay. Both stimuli evoked a significant and sustained increase of ECAR in T cells that lasted during the entire duration of the essay (Fig 6A-6C). In addition, upon stimulation the OCR/ECAR ratio drops (Fig 6D), demonstrating that T cells undergo a glycolytic switch irrespective of the stimulus employed.

We next investigated which substrate was preferentially used to generate energy during the activation of antigen-experienced T cells. To this end, we compared the TLR- and TCR-mediated activation when the “in-Seahorse” activation assay was performed in fully supplemented medium, or in medium that contained glucose only, L-glutamine only, or that was not supplemented at all. In line with earlier reports\textsuperscript{43, 44}, ECAR levels increased only in the presence of glucose, demonstrating that glucose is an important energy source for T cell function, independently of the stimulus (Fig 6E). When resting T cells were activated in medium lacking glucose we found that the oxygen consumption rate increased, indicating that respiration can substitute glycolysis as a mechanism to produce energy (Fig 6F). Interestingly, the empty medium showed similar increased OCR levels as the medium containing L-glutamine only (Fig 6F), suggesting that the switch to respiration in the absence of extracellular glucose did not rely on the presence of extracellular L-glutamine. Taken together, these data indicate that T cells can choose which metabolic process (aerobic glycolysis vs mitochondrial respiration) to employ to produce the required energy, depending on the availability of nutrients.

T cell activation through TLR requires mitochondrial respiration, but not glucose, to fuel IFN-γ production

Previous studies have found that effector CD8\textsuperscript{+} T cells rely on glucose metabolism for optimal production of IFN-γ\textsuperscript{45, 46, 47}. Here, we asked whether TLR-mediated IFN-γ production also depends on glucose availability. To this end, we activated antigen-experienced T cells with Pam3, or with OVA\textsubscript{257–264} peptide in FCS-free, glucose-free medium, and we compared their capacity to produce IFN-γ protein with that in the presence of glucose. Intriguingly, while the IFN-γ signal was significantly increased upon antigen-specific stimulation when glucose was available, we found that Pam3-mediated IFN-γ production was insensitive to changes in glucose levels in the culture medium, both in terms of percentage of IFN-γ\textsuperscript{+} T cells and of levels of IFN-γ production as determined by the Geometric Mean Fluorescence Intensity (Geo-MFI) (Fig 7A-7B). Furthermore, addition of glucose plus its antimetabolite 2-deoxy-D-glucose (2-DG) significantly reduced the production of IFN-γ in OVA\textsubscript{257–264} peptide stimulated T cells, both at high (100nM) and at low (1nM) concentrations of antigen (Fig 7B,
CD8+ T cells sense TLRs and produce IFN-γ

Fig S3E). In contrast, this treatment left IFN-γ levels unchanged in Pam3-activated T cells (Fig 7A). Therefore, we conclude that TLR-mediated T cell activation is independent of the availability of glucose levels.

Because glucose was redundant for the production of IFN-γ upon TLR2 triggering, we next examined whether L-glutamine was a critical energy source. We activated antigen-experienced T cells with Pam3, or with OVA257–264 peptide in FCS-free, L-glutamine-free medium, and we compared their responsiveness to activation in L-glutamine-supplemented medium. Interestingly, regardless of the stimulus, L-glutamine significantly increased the ability of T cells to produce IFN-γ. Again, addition of 6-diazo-5-oxo-L-norleucine (DON), a glutamine antagonist that inhibits both glutamine uptake and glutaminolysis, reduced the responsiveness of T cells back to basal levels of the empty medium (Fig 7C). Because TLR- and TCR-activated T cells already produced significant levels of IFN-γ in glucose/glutamine free media, we hypothesized that the immediate production of IFN-γ may be supported by...
Intracellular storage of nutrients that T cells acquired during the culture period.

In light of the findings that resting T cells use oxidative phosphorylation to generate ATP necessary for homeostatic functions, we examined whether this mitochondrial basal ATP production was also sufficient to fuel the initial production of IFN-γ in response to immunogenic signals. To test this, T cells were activated in the presence of oligomycin to block ATP synthesis, or of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), which uncouples ATP synthesis from the electron transport chain. Both drugs substantially affected the IFN-γ production induced by OVA peptide and Pam3, demonstrating that mitochondrial respiration is required for the initial production of IFN-γ (Fig 7D). Of note, T cells displayed the same requirements for nutrients when stimulated between day 3 and 6.
or after 15 days of rest, thereby revealing the robustness of our findings (Fig 7, and Fig S3F-3G, respectively).

Together these data show that TLR-mediated triggering of antigen-experienced T cells does not require external substrates for metabolic demands, but that internal stores of energy substrates are sufficient to promote innate production of IFN-γ in response to antigen-independent triggers.

**Discussion**

In this study, we demonstrate that CD8⁺ T cells can be directly activated by the TLR2-ligand Pam₃CysSK₄ and the TLR7-ligand R848 in an antigen-independent manner, allowing CD8⁺ T cells to respond to unrelated infections. The innate-like effector function of T cells primarily triggers the production of IFN-γ but not of cytotoxic cytokines like TNF-α, or the release of cytotoxic granules. Rather than killing infected cells, TLR-activated CD8⁺ T cells appear to exert a helper function. In an uninfected host macrophages and CD8⁺ T cells are already located in close proximity within the lymph nodes⁴⁹, which should allow for a rapid communication between the two cell types. Furthermore, a cross-talk between myeloid and lymphoid cells was shown to augment host defense in the early phase of infection⁷, ⁸, ⁴⁹. Therefore, it is tempting to speculate that TLR-mediated IFN-γ production supports this communication with myeloid cells, not only by providing costimulatory signals to effector T cells but also by bystander-activating resting T cells. In line with these findings, the fast TLR-driven release of IFN-γ by CD8⁺ T cells is sufficient to enhance the TLR-mediated activation of macrophages.

Even though bystander-activated CD8⁺ T cells are beneficial to mount an inflammatory response, they need to contain their cytotoxic capacity upon an unrelated infection to avoid immunopathology⁵⁰. We noticed that the levels of IFN-γ produced by TLR-triggered T cells are lower than upon antigen-specific activation. This restricted IFN-γ production is provided by lower production of Ifng transcripts and the failure to stabilize Ifng mRNA. This may ensure that T cell responses to unrelated infections are of short nature but sufficient to restrain pathogenic spread early on during infection, as was demonstrated in several infectious mouse models⁷, ¹⁵, ⁵¹.

While the production of Ifng transcripts upon activation of resting CD8⁺ T cells by TCR or TLR engagement depended on the activation of PI3K-Akt, only triggering with cognate antigen allowed for mRNA stabilization. Whether the difference in mRNA stabilization is determined by the strength of signaling, or a result of a qualitative difference in stimulation remains to be determined. Interestingly, mTOR is the limiting factor for protein synthesis for both stimuli, even though TLR-driven IFN-γ production was more sensitive to rapamycin. Again, this difference may be due to the strength of antigen-specific stimulation that requires high levels of rapamycin to achieve a block in protein production. Alternatively, previous studies have indicated that ng levels of rapamycin inhibit the mTORC1 complex, while high (i.e. μg) levels of rapamycin also affect the mTORC2 complex⁵₂. Hess and colleagues recently found that the immediate glycolytic switch upon TCR-mediated restimulation relies on mTORC2⁴³. Our data show that in contrast to triggering with cognate antigen, bystander activation by TLR ligands is also sensitive to low ng levels of rapamycin. Therefore, it is tempting to speculate that TLR-mediated activation may be more dependent on the
mTORC1 complex while antigen-specific stimulation primarily requires mTORC2.

A critical feature of T cells is to exert immunosurveillance so that reinfection is prevented\(^{53, 54, 55, 56}\). When T cells are in a resting state, they proliferate only at a low rate to replenish their population\(^{57, 58}\). They have a limited need for energy, which is also reflected in their reduced capacity to take up nutrients\(^{37}\). To maintain their housekeeping functions T cells produce ATP mainly through catabolic processes that breakdown amino acids, glucose and lipids via OXPHOS\(^{37, 38}\). Upon antigen-specific activation, however, T cells undergo an immediate glycolytic switch to meet the energy demands for proliferation and for optimal effector function\(^{46, 47, 59}\). Amino acids and lipids are then used for the production of molecules required to respond to infectious agents, and the generation of ATP is progressively more dependent on aerobic glycolysis\(^{60, 61}\). Here, we observe that the production of IFN-γ protein can also occur when the aerobic glycolysis is limited. We show that TLR-mediated activation of resting T cells mainly drives on ATP generated by mitochondrial respiration, independently from the delivery of exogenous nutrients. In fact, supplementing T cell cultures with glucose upon Pam3 stimulation does not increase the production of IFN-γ, and the addition of L-glutamine only has slight effects. We therefore suggest that intracellular sources of nutrients, possibly also of other amino acids such as arginine and leucine\(^{62, 63, 64}\), are sufficient to fuel the ATP production in the mitochondria, which is required for bystander activation-induced IFN-γ production by T cells. In fact, T cells take up and store high levels of metabolites during T cell priming, such as amino acids, lipids, and nucleotides\(^{65}\), and these may serve as an immediate source of energy upon T cell activation. When in a resting state, IL-7R signaling mediates the synthesis and storage of fatty acids in memory T cells, which promote T cell survival and a fast response to secondary infections\(^{66, 67}\). Memory T cells also display a substantial mitochondrial mass\(^{42}\), thereby exhibiting a greater capacity to use both OXPHOS and glycolysis to generate ATP. We therefor suggest that these features may also already be manifested in resting T cells.

In conclusion, we show that bystander activation of resting T cells through TLRs leads to significant but restrained production of IFN-γ that relies exclusively on newly synthetized transcripts and on energy generated by mitochondrial respiration. Antigen-experienced T cells are able to recognize pathogen-derived danger signals and alert innate immune cells, and this helper function does not require a glycolytic switch. The limited activation presumably acts only at short range and in the vicinity of the infection, and at the same time prevents the mobile T cells to spill out IFN-γ at distant location where no infectious pathogens are. This mechanism allows T cells to act as sensors of unrelated infections without the risk of inducing immunopathology.

**Materials and Methods**

**Mice and cell culture**

C57BL/6J mice and C57BL/6J.OT-I T cell receptor (TCR) transgenic mice (OT-I) were housed and bred in the animal department of the Netherlands Cancer Institute. All animal experiments were performed in accordance with institutional and national guidelines and approved by the Experimental Animal Committee (DEC) of the Netherlands Cancer Institute. Mouse T cells and MEC.B7.SigOVA cells were cultured in IMDM (GIBCO-BRL) supplemented
with 8% FCS, 50μM 2-mercaptoethanol, 2mM L-Glutamine, 20 U/mL penicillin G sodium, and 20μg/mL streptomycin sulfate, unless differently specified.

Bone marrow (BM) cells were obtained from femurs and tibia of C57BL/6J WT or MyD88-/- mice (kindly provided by J. den Haan), and of BALB/c WT or IFN-γR-/- mice (kindly provided by H. A. Young). BM-derived macrophages were generated by a 7 days culture in 100mm non-tissue culture treated dish at a density of 0.2×10^6 cells/ml in RPMI 1640 (GE Healthcare Life) supplemented with 10% FCS, 50μM 2-mercaptoethanol, 2mM L-Glutamine, 20 U/mL penicillin G sodium, 20μg/mL streptomycin sulfate, together with 15% L-929 conditioned medium containing recombinant M-CSF. Medium was refreshed once at day 4.

**T cell activation**

1×10^6 purified CD8+ OT-I T cells (Miltenyi CD8 isolation kit; 80-99% purity) or 1×10^6 FAC-sorted naive CD44lo CD62Lhi CD8+ OT-I T cells (BD FACSaria III Cell Sorter; 100% purity) were activated by a 20h co-culture with 0.1×10^6 pre-seeded MEC.B7.SigOVA cells per well, as described previously31. C57BL/6J total splenocytes (3×10^6 cells/well) were activated for 48h with Concanavalin A (2μg/ml, Sigma) and Interleukin 7 (rmIL-7, 1ng/ml, PeproTech). Activated T cells were harvested, removed from the stimuli and put to rest for 3-15 days in a density of 0.5x10^6/ml the presence of 10ng/ml rmIL-7. T cells were re-plated and medium was refreshed every 3-4 days.

Resting OT-I T cells were stimulated for 6h with 1nM or 100nM OVA_{257-264} peptide, or with the TLR ligands (all Invivogen) Pam$_3$CysSK$_4$ (Pam3, 1ng/ml to 10μg/ml), poly(I:C) (50μg/ml), LPS (10μg/ml), Flagellin (10μg/ml), R848 (1ng/ml to 10μg/ml), CpGA (10μg/ml), and CpGB (10μg/ml), unless differently specified.

To test possible indirect effects of IL-12, 10μg/ml IL-12 neutralizing antibody (C17.8; eBioscience) was added to resting OT-I T cells 30 min prior to TLR-dependent activation. For inhibitor experiments, 1μg/ml PI-103 (Tocris Bioscience), 10μg/ml Akti1/2, 10ng/ml to 5μg/ml Rapamycin (both Sigma Aldrich), 25ng/ml to 150ng/ml Cyclosporine A, 30μM ICS7114 (both Calbiochem), 10μM ZTSK474 (Selleckchem), or DMSO control was added to resting OT-I T cells 30 min prior to Pam3 (5μg/ml) or OVA$_{257-264}$ Peptide (1nM or 100nM) stimulation.

**Macrophage activation**

2×10^6 OT-I T cells (6 days of rest) were stimulated for 6h with 5μg/ml Pam3 or left untreated in a 24-well plate in 2ml medium per well. T cell supernatant was harvested and a total of 350μl was used to activate 0.3x10^6 macrophages per well for 5h. Macrophages were washed with PBS prior to RNA isolation.

**Flow cytometry**

Cells were washed with FACS buffer (phosphate-buffered saline [PBS], containing 1% FCS and 2mM EDTA) and labeled for 20 minutes at 4°C with the following monoclonal antibodies (all from eBioscience): anti-CD8α (S3-6.7), anti-CD8β (H35-17.2), anti-CD4 (GK1.5), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD11b (M1/70), anti-CD11c (N418), anti-IFN-γ (XMG1.2), anti-TNF-α (MP6-XT22), anti-IL-2 (JES6-5H4), anti-phospho Akt (S473, SDRNR), anti-phospho mTOR (S2448, MRRBY) and anti-phospho S6 (S235/S236, cupk43k). When necessary, cells were incubated with anti-CD16/CD32 blocking antibody (2.4G2; kind gift from Louis Boon,
Bioceros). To exclude dead cells from analysis, Near-IR (Life Technology) was added to the cells. To analyze intracellular cytokine levels, cells were fixed and permeabilized with the cytofix/cytoperm kit according to the manufacturer’s protocol (BD Biosciences). IFN-γ, TNF-α and IL-2 producing cells were gated on Near-IR<sup>neg</sup> CD8<sup>+</sup> T cells prior to analysis. To assess phosphorylation levels of Akt, mTOR and S6, activated T cells were fixed in 4% PFA and permeabilized with methanol prior to staining. Expression levels were acquired using FACS LSR Fortessa (BD Biosciences) and data were analyzed using FlowJo software (Tree Star, version 10).

**OCR and ECAR measurement**

The Seahorse XF-96 metabolic extracellular flux analyzer (Seahorse Bioscience) was used to measure OCR (in pmol/min) or ECAR (in mpH/min). After 7 days of rest, CD8<sup>+</sup> OT-I T cells were resuspended in nutrient-free unbuffered DMEM medium (Sigma-Aldrich) that was supplemented with 16mg/L phenol red, 1.85g/L NaCl, and 2M NaOH. The nutrient-free unbuffered DMEM medium was used with or without the presence of 25mM glucose, 1mM Na Pyruvate and 2mM L-Glutamine (all Sigma-Aldrich). 0.3×10<sup>6</sup> cells/well were plated into Seahorse cell plates coated with poly-lysine (Sigma-Aldrich) to enhance T cell attachment. Pam3, OVA<sub>257-264</sub> peptide, or medium (used as negative control) were directly applied to plated cells by using the instrument’s multi-injection ports. Experiments with the Seahorse system were performed with the following assay conditions: 2 min mixture and 3 min measurement. Data were measured in 6-plo or 9-plo and analyzed using Wave software (Seahorse Bioscience).

**Glucose, glutamine and mitochondrial respiration-dependency assay**

For glucose-dependency experiments, resting OT-I T cells were washed once in serum-free glucose-free RPMI 1640 medium (Life Technologies) and then plated in the same medium with or without 25mM glucose (Sigma-Aldrich). For inhibition of glucose usage, 10mM 2-deoxy-D-glucose (2-DG; Sigma-Aldrich) was added to the stimulation.

For glutamine-dependency experiments, resting OT-I T cells were washed once in serum-free L-glutamine-free RPMI 1640 medium (Life Technologies) and then plated in the same medium with or without 2mM L-Glutamine (Sigma-Aldrich). For inhibition of glutamine usage, cells were cultured in the presence of 500μM diazo-5-oxo-L-norleucine (DON; Sigma-Aldrich). For mitochondrial respiration-dependency experiments, resting OT-I T cells were activated in the presence of 15μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), or 10μM oligomycin (Sigma-Aldrich).

**Quantitative PCR analysis**

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative Real-Time PCR was performed with SYBR green and a StepOne Plus RT-PCR system (both Applied Biosystems). The sequences of primers used are listed in Supplementary Table 1. Reactions were performed in triplicate. C<sub>T</sub> values were normalized to L32 levels.

When mRNA stability was studied, resting T cells were treated with 10μg/ml Actinomycin D (Sigma-Aldrich) for indicated time points upon 1h up to 3h of activation.
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Statistical analysis
Results are expressed as mean ± SD. Statistical analysis between groups was performed with GraphPad Prism 6, using paired or unpaired 2-tailed Student t-test when comparing 2 groups, or 1-way ANOVA test with Dunnett’s multiple comparison when comparing > 2 groups. P values < 0.05 were considered to be statistically significant.

Author contributions
F.S. and M.C.W. designed research; F.S., A.G., and D.C. performed experiments; F.S. and M.C.W. analyzed data and wrote the manuscript.

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Disclosures
The authors have no financial conflict of interest.
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Supplementary Figures

Supplementary Figure 1: TLR-dependent production of IFN-γ by T cells

(A) Intracellular IFN-γ staining was performed on MACS-enriched CD8+ OT-I T cells directly post selection (ex vivo), after activation with MEC.B7.SigOVA cells for 20h or at indicated time points of rest in the absence of antigen. T cells were left untreated, or were incubated for 6h with 1μg/ml brefeldin A (BFA), in the presence or absence of 1μg/ml OVA257-264 peptide or the irrelevant SV40560-568 peptide. Numbers in the upper right corner indicate the percentage of IFN-γ+ CD8+ T cells. (B) MACS-selected T cells (rested for 3 days) were pre-incubated for 30 min with 10μg/ml IL-12 neutralizing antibody or left untreated (-), and then stimulated for 6h with Pam3 and R848 in the presence or absence of the IL-12 neutralizing antibody. IFN-γ production was measured by flow cytometry. (C-D) FACS-sorted naive CD44low CD62Lhi CD8+ OT-I T cells were activated with MEC.B7.SigOVA cells for 20h and removed from antigenic stimuli for indicated time points. (C) Dot plots represent IFN-γ and TNF-α (upper panel), and IFN-γ and IL-2 production (lower panel) of T cells (rested for 3 days) that were stimulated for 6h with 5μg/ml Pam3 or 10μg/ml R848. (D) Intracellular IFN-γ staining of T cells that were activated with Pam3 and R848 upon 6 days, or 15 days of rest.
Supplementary Figure 2: Pam3 stimulation of IFN-γR−/- macrophages

BM derived-macrophages from Balb-c WT and IFN-γR−/- mice were cultured for 5h with 5μg/ml Pam3 or left untreated. (A) Tnfa and (B) Il10 mRNA expression was analyzed by RT-PCR. Data are pooled from 2 independently performed experiments.
Supplementary Figure 3: TCR-dependent IFN-γ production is controlled by both mTORC1 and mTORC2 pathways, and is supported by glycolysis.

(A) Dot plots represent Akt, mTOR and S6 phosphorylation 30 minutes after T cell reactivation with 5μg/ml Pam3 or 100nM OVA257-264 peptide. When indicated, cells were pretreated for 30 minutes with ZTS inhibitor. (B) Intracellular IFN-γ staining of CD8+ T cells that were activated for 6h with 5μg/ml Pam3, 0.1nM, 1nM or 100nM OVA257-264 peptide or were left unstimulated. (C) Resting T cells were incubated for 30 min with indicated concentrations of rapamycin or left untreated prior to reactivation with 1nM OVA257-264 peptide. Graph indicates percentage of IFN-γ producing cells relative to cells that were reactivated in the absence of rapamycin. Data are pooled from 4 independent experiments ± SD [one-way ANOVA with Dunnett’s multiple comparison to untreated control; **p<0.005; ***p<0.0001]. (D) Akt, mTOR and S6 phosphorylation of T cells reactivated for 30 minutes with low amount (1nM) of OVA257-264 peptide. (E) T cells were activated for 6h with 1nM OVA257-264 peptide in FCS-free, glucose-free RPMI that was supplemented with 25mM glucose, or with 25mM glucose plus 10mM 2-DG, or left unsupplemented. [one-way ANOVA with Dunnett’s multiple comparison to glucose condition; **p<0.005]. (F) Glucose-dependency, (G) glutamine-dependency and mitochondrial respiration-dependency assays were performed when T cells were rested for 15 days and then stimulated for 6h with 5μg/ml Pam3 or 100nM OVA257-264 peptide. [one-way ANOVA with Dunnett’s multiple comparison to negative condition; *p<0.05; **p<0.005].
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### Supplementary Table 1: Primers used for RT-PCR

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<th>Gene (mouse)</th>
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