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Research In-Press Preview **Immunology**

Induction of memory CD8 T cells is important for controlling infections such as malaria HIV/AIDS, and for cancer immunotherapy. Accurate assessment of antigen (Ag)-specific CD8 T-cells is critical for vaccine optimization and defining correlates of protection. However, conditions for determining Ag-specific CD8 T-cell responses ex-vivo using ICS may be variable, especially in humans with complex antigens. Here, we used an attenuated whole parasite malaria vaccine model in humans and various experimental infections in mice to show that the duration of antigenic stimulation and timing of brefeldin A (BFA) addition influences the magnitude of Ag-specific and bystander T cell responses. Indeed, following immunization with an attenuated whole sporozoite malaria vaccine in humans, significantly higher numbers of IFN-γ producing memory CD8 T-cells comprised of antigen specific and bystander responses were detected by increasing the duration of Ag-stimulation prior to addition of BFA. Mechanistic analyses of virus-specific CD8 T-cells in mice revealed that the increase in IFNg producing CD8 T-cells was due to bystander activation of Ag-experienced memory CD8 T-cells, and correlated with the proportion of Ag-experienced CD8 T-cells in the stimulated populations. Incubation with anti-cytokine antibodies (ex. IL-12) improved accuracy in detecting *bona-fide* memory CD8 T-cell responses suggesting this as the mechanism for the bystander activation. These data have important implications for accurate assessment of immune responses generated by vaccines intended to […]

Bystander responses impact accurate detection of murine and human antigen-specific CD8 T cells

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Abstract

Induction of memory CD8 T cells is important for controlling infections such as malaria HIV/AIDS, and for cancer immunotherapy. Accurate assessment of antigen (Ag)-specific CD8 T-cells is critical for vaccine optimization and defining correlates of protection. However, conditions for determining Ag-specific CD8 T-cell responses ex-vivo using ICS may be variable, especially in humans with complex antigens. Here, we used an attenuated whole parasite malaria vaccine model in humans and various experimental infections in mice to show that the duration of antigenic stimulation and timing of brefeldin A (BFA) addition influences the magnitude of Ag-specific and bystander T cell responses. Indeed, following immunization with an attenuated whole sporozoite malaria vaccine in humans, significantly higher numbers of IFN–γ producing memory CD8 T-cells comprised of antigen specific and bystander responses were detected by increasing the duration of Ag-stimulation prior to addition of BFA. Mechanistic analyses of virus-specific CD8 T-cells in mice revealed that the increase in IFN–γ producing CD8 T-cells was due to bystander activation of Ag-experienced memory CD8 T-cells, and correlated with the proportion of Ag-experienced CD8 T-cells in the stimulated populations. Incubation with anti-cytokine antibodies (ex. IL-12) improved accuracy in detecting *bona-fide* memory CD8 T-cell responses suggesting this as the mechanism for the bystander activation. These data have important implications for accurate assessment of immune responses generated by vaccines intended to elicit protective memory CD8 T-cells.

Introduction

CD8 T cells have a role in mediating protection in humans against diverse pathogens impacting public health such as HIV, influenza, and *Plasmodium*, the causative agent of malaria, and tumors (1-5). The critical factors for mediating protective immunity by T cells include the magnitude, quality, breadth, and location. Indeed, subjects with greater numbers of memory CD8 T cells are better protected against infection (6, 7). Therefore, the development of preventive and therapeutic vaccines against infections and tumors requires a precise understanding of how to generate and accurately assess CD8 T cell responses.

There are now a variety of techniques to measure CD8 T cell responses, each with strengths and weaknesses. Peptide-MHC tetramer staining allows for an accurate enumeration of epitope-specific cells within the host and the evaluation of their phenotypic traits, but provides limited information on the functionality of the T cell response. Additionally, peptide-MHC tetramer analyses require knowledge of both the exact T cell epitope and MHC allele, which vary considerably between individuals, making assessment of the antigenic breadth of response resource intensive. Antigen-stimulated detection of T cells by ICS, in contrast, allows for assessment of T cell responses in an MHC agnostic manner since stimulation of T cells occurs through autologous antigen (Ag) presentation. However, a limitation of the ICS assay, in which overlapping peptides are often used to stimulate T cells, is that the exact T cell epitope is not determined, and thus, the breadth of the host responses is not as precisely defined. Additionally, while ICS assays can provide data on the functional and phenotypic traits of responding cells, cells typically require stimulation for greater than 6 hours, during which time the cell phenotype of activated cells may change.

While alternative methods for detecting Ag-specific cells are used by some groups, such as activation-induced expression of CD137 (8), IFN–γ is the canonical gold-standard cytokine for

ICS-based enumeration of Ag-specific CD8 T cells. In a review of the literature, we determined that methods of conducting ICS, including length of incubation and timing of BFA (a golgi-disrupting compound used to prevent secretion of cytokines) addition, vary widely. Most often, ICS stimulation occurs for between 5 and 24 hours, and BFA is added several hours after the beginning of stimulation (4, 6, 9-39). While delaying addition of BFA may be necessary with certain types of antigen stimulations such as using whole pathogens rather than peptides to allow for Ag processing and presentation on MHC needed to drive cytokine production by CD8 T cells, delayed BFA addition may also allow for the release of cytokines into the culture media if using stimuli that have innate stimulatory activity. Inflammatory cytokines such as IL-12 could enhance IFN–γ production by Ag-specific CD8 T cells that respond to antigens used for stimulation, but also by memory T cells that are specific for other antigens (bystander activation). This is supported by evidence that Ag-experienced effector or memory CD8 T cells can be induced to produce IFN–γ not only in response to cognate Ag, but also in a bystander manner that is solely driven by inflammatory cytokines (40-42). Thus, we sought to understand how variations in the conditions of ICS impact the accurate accounting of infection- or vaccine-induced Ag-specific CD8 T cells. These data have implications for improving vaccine design and accurately assessing correlates of CD8 T cell mediated protection.

Results

The frequency of IFN–γ producing CD8 T cells detected in humans following vaccination is increased with increased time of ex-vivo Ag stimulation

The potency and protective capacity of a vaccine can be evaluated partly based upon the magnitude of the induced memory CD8 T cell population. To begin to address the impact of varying the assay conditions on enumeration of Ag-specific CD8 T cell memory, we assessed the frequency of Ag-specific memory CD8 T cells following vaccination of human subjects with

an attenuated whole sporozoite malaria vaccine (4, 6, 43). Employing a peptide stimulation assay for this vaccine would be technically challenging due to MHC polymorphism and the large number (>2000) of potential antigens presented to the immune system. Therefore, we utilized a previously developed assay where stimulation of CD8 T cells was achieved by incubating PBMCs from vaccinated subjects with the vaccine itself, which consists of aseptic, purified, irradiated, metabolically-active *Plasmodium falciparum* sporozoites (PfSPZ), and where 12-hour stimulation was more sensitive than 6-hour stimulation (4). Here, we extended these data and incubated PBMCs from a vaccinated or nonimmunized subject with PfSPZ for 12, 16, 20, and 24 hours, with BFA being added for the final four hours. While the nonimmunized subject had no IFN–γ production above background for the duration of stimulation, the percentage of CD8 T cells that produced IFN–γ in the PfSPZ-vaccinated subject increased substantially over time (Figure 1A), suggesting that duration of ICS increased the detection of total IFN–γ producing CD8 T cells.

Addition of BFA is used in ICS assays to block release of IFN–γ within the T cell, thereby improving the sensitivity of the response. However, BFA also could inhibit efficient Ag-processing and presentation of PfSPZ (44). To determine if timing of BFA addition impacted the detection of IFN–γ producing CD8 T cells, cells were stimulated with PfSPZ for a total of 24 hours, with BFA being added after the first 8, 16, or 20 hours of incubation. A substantially higher percentage of CD8 T cells producing IFN–γ was detected when BFA was added 16 or 20 hours after beginning stimulation (Figure 1B), indicating that the timing of BFA addition strongly influences the frequency of IFN–γ producing CD8 T cells detected.

Delayed addition of BFA would allow for soluble factors including inflammatory cytokines to be released into the culture, and potential bystander activation of Ag-experienced effector and memory CD8 T cells. Thus, it is unclear if enhanced detection of IFN–γ producing CD8 T cells observed by increasing incubation time or by delaying addition of BFA was due to

increased sensitivity in detecting *Plasmodium*-specific CD8 T cells, and/or by triggering IFN–γ-production by non-malaria Ag-experienced CD8 T cells.

Delayed addition of BFA leads to bystander activation of Ag-experienced CD8 T cells

To further determine how ICS conditions altered detection of Ag-specific CD8 T cells, we used a well-defined mouse model that allows precise detection of CD8 T cells of known Ag-specificity. Mice (B6, Thy1.2) received adoptive transfer of naïve TCR-transgenic GP_{33}-specific P14 cells (Thy1.1), before LCMV-Armstrong infection (Figure 2A), and memory P14 cells were detected by Thy1.1 expression. Additionally, infection of B6 mice with LCMV elicits large Ag-specific CD8 T cell responses that recognize LCMV-derived GP_{33} and NP_{396} epitopes (45), and we used peptide stimulated ICS to detect cytokine producing memory CD8 T cells responding to GP_{33} and NP_{396} peptides (Figure 2B-D). Mice generated in this manner contain endogenous (Thy1.1 neg- blue gates) CD8 T cells that consist of naive cells and Ag-specific cells that recognize GP_{33} and NP_{396} epitopes as well as additional LCMV-derived epitopes, and Ag-experienced P14 cells (Thy1.1 pos- red gates) that recognize GP_{33}, but not NP_{396} peptides. Addition of P14 cells allows for detection of true Ag-specific responses (in response to GP_{33} peptide) and bystander responses (in response to NP_{396} peptide), while IFN-γ production by endogenous CD8 T cells, due to mixed epitope specificities of this population, could represent either true Ag-specific responses or responses that include both Ag-specific and bystander responses. To highlight this, as can be seen in Figure 2B-D, we present detection of IFN-γ producing cells following ICS gated on total lymphocytes (lymphocyte gate), total CD8 T cells with identification of endogenous (blue gates) or P14 (red gates) cells based on Thy1.1 expression (CD8 gate), or total P14 cells (Thy1.1+/CD8+ (P14) gate).

Of note, since detection of IFN–γ producing CD8 T cells is impacted by peptide concentration and number of cells plated, we first identified conditions that allow for maximal detection of bona fide Ag-specific CD8 T cells (ex. $2x10^6$ cells/well and 200 nm peptide concentration) (Supplemental Figure 1, A and B). Inflammatory cytokines can potentially cause bystander activation of Ag-experienced CD8 T cells when BFA is not present during the entire incubation (40-42, 46), so we first sought to determine if timing of BFA addition impacted detection of IFN–γ producing CD8 T cells following specific peptide stimulation. Interestingly, between a ~1.5- to 2-fold increase in the percentage of endogenous CD8 T cells producing IFN–γ in response to GP_{33} or NP_{396} peptide was observed when BFA was added for the last hour (7+1) compared to when it was present during the entire incubation (0+8) (Figure 2, B-D CD8 gate - blue boxes and Figure 2E). Similar results were observed for LCMV immune mice that did not receive P14 cells (Supplemental Figure 1, C and D).

A similar percentage of P14 cells produced IFN–γ following GP_{33} peptide stimulation regardless of timing of BFA addition (Figure 2C P14 gate), suggesting that delayed addition of BFA does not impact IFN–γ production of *bona fide* Ag-specific CD8 T cells. To determine whether increases in IFN–γ producing CD8 T cells that occurred with delayed BFA addition were due to bystander activation, we examined responses of 'sensor' GP_{33}-specific Thy1.1 P14 cells in response to NP_{396} peptide stimulation. While few IFN–γ producing P14 cells were detected following stimulation with NP_{396} peptide in the presence of BFA for the entire incubation, 25-30% of memory P14 cells produced IFN–γ in response to NP_{396} peptide when BFA was not present during the whole incubation time (Figure 2D red boxes). Analyses of T cell responses by ICS in human samples often rely on stimulation for greater than 8 hours, with addition of BFA before the last hour of incubation. To determine if bystander responses contribute to IFN–γ producing cells detected for incubation times of greater length and when BFA is added earlier in the

culture, we stimulated splenocytes from LCMV immune mice that contained memory P14 cells with NP_{396} peptide for 8 hours and added BFA after 4 or 7 hours, or for 12 hours and added BFA after 4, 8, or 11 hours. Bystander responses by P14 cells could be detected when BFA was added prior to the last hour and when cells were stimulated for 8 or more hours, and bystander responses increased with greater length of stimulation in the absence of BFA (Supplemental Figure 2). Thus, delayed addition of BFA in both the mouse and human models of viral and malaria specific CD8 T cells can result in bystander activation of Ag-experienced CD8 T cells leading to inflation in frequencies and numbers of Ag-specific CD8 T cells detected.

Contribution of bystander IFN–γ activation is dependent upon CD8 T cell pool composition and length of stimulation

Ag-experienced CD8 T cells can undergo bystander activation and produce cytokines such as IFN–γ while naïve CD8 T cells cannot (40, 46). Of note, the representation of Ag-experienced cells within the CD8 T cell pool for human subjects can vary widely based on age and history of previously encountered infections (47, 48). To determine whether the composition of Ag-experience in the CD8 T compartment dictated frequencies of IFN–γ producing CD8 T cells, we manipulated the number of Ag-experienced cells among splenocytes by mixing splenocytes from LCMV-immune mice with graded numbers of sorted memory P14 'sensor' cells to achieve different ratios of naïve (CD11a low/CD8 hi) to memory (CD11a hi/CD8 low) CD8 T cells (49, 50) (Figure 3, A and B). Again, few IFN–γ producing P14 cells were detected following stimulation with NP_{396} peptide when BFA was present for the entire incubation, and a similar percentage of CD8 T cells producing IFN–γ in response to NP_{396}-peptide stimulation was observed regardless of numbers of P14 cells present (Figure 3, C and D, (0+8 group)). However, when BFA was not present for the entire incubation, the percentage of IFN–γ producing CD8 T cells in response to NP_{396}-peptide stimulation increased with increasing

numbers of 'sensor' P14 cells, and this was due to increased representation of P14 cells rather than elevation in the frequency of activated bystander P14 cells (Figure 3, C and D, (7+1 group)). Thus, the contribution of bystander activated cells to the IFN–γ producing CD8 T cell population was dependent upon the representation of Ag-experienced cells within the CD8 T cell compartment. These data suggested that the increase in the frequency of vaccine targeted memory CD8 T cells might be more pronounced in older subjects and/or subjects with substantial history of pathogen exposures that possess more previously activated CD8 T cells.

The duration of antigen-stimulation *ex vivo* for experiments involving human subjects varies, but in most instances stimulation times are between 5 and 24 hours (4, 6, 9-39). To determine if the duration of stimulation contributed to the degree of bystander IFN–γ detected when BFA is not present for the entire incubation, splenocytes from an LCMV immune mouse were incubated with GP_{33} or NP_{396} peptides for 5, 8,16, or 24 hours with BFA present for the entire incubation or for the final hour of incubation. Regardless of length of incubation, a greater percentage of IFN–γ producing CD8 T cells was detected following stimulation with GP_{33} (Figure 4, A and B) or NP_{396} (Figure 4, C and D) peptides when BFA was added for only the last hour of incubation. However, increased detection of IFN–γ producing CD8 T cells was most pronounced when samples were incubated for greater than 5 hours. Similarly, bystander activated P14 cells were detected in response to NP_{396}-peptide stimulation when BFA addition was delayed for any length of incubation tested, but percentages of bystander activated P14 cells detected were greater when samples were incubated for greater than 5 hours (Figure 4E). These data suggested that bystander activation occurred and contributed to the frequency of IFN–γ producing CD8 T cells detected when BFA is not present for the entire incubation across the spectrum of incubation times used to conduct ICS. However, contribution of bystander activated cells to IFN–γ producing cells detected is likely to increase with greater lengths of incubation.

Delayed addition of BFA leads to bystander activation of Ag-experienced CD8 T cells following stimulation with whole pathogen

Application of peptide-stimulated ICS assays across individual human subjects assessing responses to complex pathogens with many antigens is difficult due to differences in HLA haplotype, thus, stimulation of human samples is often achieved by exposing samples to whole pathogens (Figure 1), or through the use of libraries of long overlapping peptides, methods that may require a period of BFA-free culture to allow for optimal Ag processing and presentation or cross presentation. To determine if bystander activated cells contributed to the frequency of IFN–γ producing cells detected after stimulation with whole pathogens, splenocytes from an LCMV immune mouse ('sensor' cells) were incubated with CFSE labeled splenocytes that were either pulsed with GP_{33} peptide or infected with VacV expressing cognate Ag (VacV–GP_{33}) or an irrelevant Ag (VacV-OVA) (Figure 5A). When BFA was present throughout the incubation period, IFN–γ production from the LCMV immune sensor cells was observed in response to spleen cells pulsed with GP_{33} peptide or infected with VacV–GP_{33} and the percentage of IFN–γ producing CD8 T cells detected increased with increasing numbers of stimulator cells added to the culture (Figure 5B). IFN–γ production was not observed in response to splenocytes infected with VacV-OVA (Figure 5C, bottom).

Interestingly, an increased percentage of IFN–γ producing sensor cells were detected following incubation with greater numbers of GP_{33} pulsed or VacV–GP_{33} infected stimulator cells and when addition of BFA was delayed for longer periods (Figure 5C top and middle). These data suggested that length of incubation with BFA impacted detection of IFN–γ producing CD8 T cells following stimulation with pathogen infected splenocytes. However, because we could not determine if our stimulation conditions resulted in IFN–γ production by bona fide Ag-specific or

any Ag-experienced CD8 T cells, we were unable to conclude if delayed addition of BFA resulted in increased detection of true Ag-specific CD8 T cells, or if increased percentages of IFN–γ producing CD8 T cells detected was due to bystander activation of memory CD8 T cells. To address this, we incubated sensor cells from LCMV P14 chimera immune mice with stimulator cells that were either pulsed with NP_{396} peptide or infected with VacV expressing LCMV nucleoprotein (VacV-NP). An increased percentage of IFN–γ producing cells was detected following stimulation with peptide pulsed or VacV-NP infected cells when BFA was not present for the whole incubation time (Figure 5, D and E). Importantly, in the same samples, GP_{33}-specific P14 memory CD8 T cells produced IFN–γ in response to VacV-NP infected splenocytes, strongly suggesting antigen-independent bystander activation. Thus, stimulation with whole pathogens, while not as potently as peptide-stimulation, also resulted in bystander CD8 T cell activation, and the potential for inaccurate accounting of pathogen/vaccine specific CD8 T cell responses.

Blocking inflammatory cytokines limits bystander activation of CD8 T cells

Bystander IFN–γ production by effector or memory CD8 T cells can be stimulated by hundreds of inflammatory cytokine combinations (42). As an example, a large percentage of endogenous and P14 memory CD8 T cells derived from LCMV immune P14 chimera mice produced IFN–γ in response to IL-12 and IL-18, IL-12 and TNF-α, or IL-12 and IL-15 stimulation alone, but only when BFA was not present during the entire incubation (Figure 6A and Supplemental Figure 3 A and B). Similarly, addition of IL-12 and IL-18 significantly increased the frequency of IFN–γ producing CD8 T cells even in the presence of peptide (GP_{33} – Fig 6B; NP_{396} – Figure 6C) stimulation, but only if BFA addition was delayed. Of note, addition of IL-12 and IL-18 (one of the most potent cytokine combinations that leads to bystander activation) (42) did not increase the number of peptide stimulated IFN–γ producing cells (GP_{33} or NP_{396} – Figure 6D) if BFA was

present all the time (0+8 group). Furthermore, as described previously, human PBMCs incubated with IL-12 and IL-18 also produced IFN–γ (51-53), but only when BFA was not present during the entire incubation (Supplemental figure 3 C and D). This suggested that human samples also may be susceptible to inflammation-driven bystander responses, similar to those observed in mice during ICS when BFA is not present for the entire incubation. Thus, accurate detection of Ag-specific human CD8 T cells by ICS may be influenced by timing of BFA addition.

These data also suggest that bystander responses elicited in response to Ag-stimulation when BFA is not present in culture for the entire incubation require intact golgi function. Two possible explanations for the absence of bystander responses in the presence of BFA for the entire culture, then, are 1) that BFA prevents cytokine secretion that elicits bystander responses, or 2) that BFA prevents transport of cytokine receptors to the cell surface, blocking the ability of cells to respond to inflammatory cues. It is also possible that both mechanisms contribute to bystander responses elicited when addition of BFA is delayed. Data presented in Figure 2 and Supplemental Figure 3 suggests that cytokines secreted in response to cognate Ag can drive bystander responses when addition of BFA is delayed, as adding non-cognate Ag alone is able to induce bystander responses (7+1, Thy1.1+/CD8+ (P14) gates). Additionally, when we analyzed expression of cytokine receptor components we found that transcript levels of *Il12rb2*, a signaling component of the IL-12 receptor complex that activates STAT4 signaling and whose expression is regulated by inflammatory cytokines (54), and *Tnfrs1b*, which binds TNFα and activates NF-κB and MAPK pathways (55), were increased in sorted P14 cells that were activated in a bystander manner in ICS cultures stimulated with NP_{396} peptide where addition of BFA was delayed (Supplemental Figure 4, A and B). Transcript levels of *Ifngr1* and *Ifngr2*, which bind IFN-γ and activates STAT1 pathways (56), were not impacted by delayed addition of

297 BFA in P14 cells (Supplementary Figure 4B), suggesting that the absence of BFA does not
298 sensitize cells capable of undergoing bystander responses to IFN-γ mediated signaling.
299 However, since IFN-γ receptors are expressed on the surface of nearly all cells, IFN-γ may be
300 acting on other cells in ICS cultures that play a role in driving bystander responses. Thus,
301 delayed addition of BFA during ICS is likely to elicit bystander responses through the
302 combinatorial effects of allowing for secretion of inflammatory cytokines that drive bystander
303 responses into culture media, and by allowing for export of inflammatory cytokine receptors to
304 the surface of Ag-experienced cells, which enhances sensitivity to inflammatory cytokines.
305
306 A further suggestion from the data presented in Figure 6 is that cytokine blockade during
307 stimulation could enhance the fidelity of detecting bone fide antigen-specific CD8 T cells by the
308 ICS assay. To test this, splenocytes from LCMV chimera P14 mice were incubated with NP_{396}
309 peptide for 8 hours in the presence of BFA for the entire incubation or for the final hour, with or
310 without anti-IL-12, -IFN–γ and/or -TNF-α blocking Abs. While incubation of splenocytes in the
311 absence of BFA led to bystander IFN–γ production and increased percentages of IFN–γ
312 producing CD8 T cells, addition of a cocktail of cytokine blocking Abs was maximally effective at
313 limiting bystander responses, and addition of blocking Abs to individual cytokines, to varying
314 degrees, reduced the percentages of endogenous (Figure 7A) or P14 cells (Figure 7B)
315 producing IFN–γ in a bystander manner, thus improving the overall accuracy in detecting Ag-
316 specific memory CD8 T cells. In summary, these data suggest that blocking inflammatory
317 cytokines during stimulation can reduce the contribution of bystander activated cells to IFN–γ
318 producing Ag-specific CD8 T cells detected by ICS, and may provide for a more accurate
319 estimation of Ag-specific CD8 T cells using different ICS protocols.
320

Bystander activated human CD8 T cells contribute to IFN–γ-producing cells detected following stimulation with whole pathogens

The data in Figure 5 from the mouse model showed that stimulation with whole pathogens could lead to bystander activation of CD8 T cells, and the data in figure 1 from humans showed that there was a higher percentage of IFN–γ producing cells detected in PfSPZ vaccinated human subjects following ICS of longer duration and when BFA was added later in the stimulation. To determine if bystander activation of CD8 T cells contributed to the pool of IFN–γ producing CD8 T cells detected in human subjects, PBMCs from PfSPZ- vaccinated subjects were labeled with CFSE and individually combined at a ratio of 9:1 with PBMCs obtained from the same subjects prior to vaccination (Figure 8A). This design allows for detection of bystander responses, as any IFN-γ producing cells in the pre-vaccine population of cells could only be due to non-cognate Ag driven responses. Cells were then incubated with PfSPZs for 12, 16, or 20 hours in the presence of BFA for the last 4 hours. While IFN–γ producing CD8 T cells were low to undetectable when pre-vaccine PBMCs were cultured in the absence of post-vaccine PBMCs, a significant percentage of pre-vaccine PBMCs produced IFN–γ when cultured with post-vaccination PBMCs (Figure 8B). These data showed that, under conditions of stimulation used most commonly for detection of CD8 T cells by the ICS assay from subjects that receive whole sporozoite vaccine (57, 58), bystander activation of human CD8 T cells can confound the enumeration of bona fide pathogen/vaccine induced memory CD8 T cells.

Of note, design of whole pathogen stimulation assays for mice shown in figure 5, where infection of stimulator splenocytes occurred prior to mixing with sensor cells of interest, was different than for the human assays shown in figure 8, where whole pathogen was added directly to cells of interest at the initiation of culture. To determine if bystander responses in mice following whole pathogen stimulation also contributed to IFN–γ producing cells detected when whole pathogens were added directly to samples being analyzed, we designed

346 experiments shown in Supplemental Figure 5A and D. With two different models, activation of
347 P14 cells following stimulation with bacterium *Listeria monocytogenes* (LM-OVA/B8R)
348 (Supplemental Figure 5B) and activation of OT-I cells following stimulation with Vaccinia virus
349 (VacV–GP_{33}) (Supplemental Figure 5E), bystander responses contributed to IFN–γ producing
350 cells detected. Furthermore, addition of cytokine blocking Abs reduced the contribution of
351 bystander P14 responses detected (Supplemental Figure 5C).
352 Additionally, to determine if bystander responses in mice influenced evaluation of CD8 T
353 cell responses elicited following experimental malaria vaccination, we performed ICS using
354 GAP50 peptide, which is a *Plasmodium berghei*-derived epitope for which naïve B6 mice
355 possess a large Ag-specific naïve CD8 T cell repertoire (59), with splenocytes from mice that
356 were inoculated with radiation attenuated *Plasmodium berghei* sporozoites, and that contained
357 memory P14 cells generated in response to prior infection with LCMV (Supplemental Figure
358 6A). The size of the CD8 T cell response detected, and contribution of bystander activated cells
359 to IFN–γ producing cells detected increased with increasing length of incubation in the absence
360 of BFA (Supplemental Figure 6 B and C). Thus, modified mouse models recapitulate the
361 findings observed with human cells, suggesting that bystander responses can contribute to
362 IFN–γ producing cells detected when addition of BFA is delayed.
363 Lastly, to determine if addition of cytokine blocking Abs may be useful in limiting
364 contribution of bystander responses to human Ag-specific CD8 T cells detected by ICS when
365 addition of BFA is delayed, we performed ICS with human PBMCs obtained at the University of
366 Iowa DeGowen Blood Center using peptide pools containing cytomegalovirus (CMV) and
367 Epstein-Barr virus (EBV) derived epitopes. Percentages of IFN-γ producing cells detected were
368 elevated when addition of BFA was delayed (Supplemental Figure 7A and B- black dots
369 compared to white dots), suggesting that bystander responses were contributing to Ag-specific
370 cells detected when BFA addition was delayed. However, percentages of IFN-γ producing cells

371 detected were not significantly different between samples incubated in the presence of BFA
372 from the beginning of stimulation and for samples for which addition of BFA was delayed but for
373 which cytokine blocking Abs were added to ICS cultures (Supplemental Figure 7A and B- black
374 dots compared to red dots). These data suggest that addition of cytokine blocking Abs during
375 ICS may be useful for accurate assessment of numbers of true Ag-specific human CD8 T cells
376 when using ICS.
377
378 **Discussion**
379 The frequency and phenotype of Ag-specific memory CD8 T cells present prior to infection
380 impact the host's ability to fight pathogenic microorganisms. Therefore, accurately identifying
381 Ag-specific memory CD8 T cells generated in response to vaccination will facilitate vaccine
382 development, for example, through accurate assessment of the immune correlates of protection.
383 Additionally, assays that seek to further characterize the CD8 T cell response, for example, by
384 determining expression of phenotypic markers on IFN–γ-producing cells by multi-parameter flow
385 cytometry or by sorting activated cells and performing single-cell RNA sequencing, may be
386 misled by incorrect identification of bona-fide Ag-specific CD8 T cells.
387
388 Here we have shown that, when using ICS to detect CD8 T cell responses, bystander activation
389 of Ag-experienced CD8 T cells can occur when BFA is not present for the entire period of
390 stimulation. Bystander activation was seen in both mouse and human models of viral and
391 malaria infection as well as bacterial infection in mice, and was influenced by the length of
392 stimulation with peptides and whole pathogens. Of note, the contribution of bystander activated
393 cells to the IFN–γ producing CD8 T cell population depended on the frequency of Ag-
394 experienced CD8 T cells in the sample. Thus, the over-representation of Ag-specific CD8 T cells
395 when ICS is performed without BFA for the entire incubation period will vary depending on the

individual examined. However, delaying addition of BFA in human samples may be unavoidable when the antigen sources are whole pathogens/proteins that require undisrupted cell machinery for efficient antigen processing and presentation or cross presentation. In these cases, addition of cytokine blocking Abs to ICS cultures may aid in minimizing bystander responses and result in greater accuracy for detection of true Ag-specific responses.

This study does have limitations. There are a number of additional parameters that may vary in ICS protocols that have the potential to impact readout of IFN-γ producing cells detected such as number of T cells in culture, representation of Ag-presenting cells, media used, and instrumentation. This is not an exhaustive list, and we were unable to test the impact of every parameter on ICS readout. Furthermore, while we did examine a number of different incubation lengths and timings of BFA addition, we did not exhaustively test how length of incubation and timing of BFA addition impact bystander activation during ICS culture. We were able to detect bystander responses across a range of incubation times from 5 to 24 hours with addition of BFA as early as 4 hours after initiation of ICS (Figure 4 and Supplemental Figure 2). However, bystander responses became magnified with incubations of greater length and with addition of BFA at later times after onset of culture. Thus, choosing incubation times of shorter duration with addition of BFA at earlier times, when allowed for based on nature of stimulation, may be an additional method to improve accuracy in detection of true Ag-specific responses by ICS.

If ICS assay is providing inaccurate estimates of memory CD8 T cells present within the host, are other techniques available that might provide a more accurate estimate? An alternative assay for measuring Ag-specific T cell responses, the ELISpot assay, is similarly compromised by bystander activation. While ELISpot is generally considered to be more sensitive than ICS, and thus better-suited for detection of rare Ag-specific cells (60), like ICS, it relies on detection of IFN–γ producing CD8 T cells following incubation with cognate peptide. Additionally, ELISpot

assays commonly have incubation periods lasting for 18-48 hours, and such long incubation periods are likely to further exacerbate the bystander activation phenomenon.

Peptide-MHC tetramer staining is also used to measure Ag-specific T cell responses. Unlike ICS conducted following stimulation with antigenic peptide pools or whole pathogens, tetramer staining must be tailored to individual subjects due to differences in MHC haplotypes among individuals (61). Therefore, it can be more logistically challenging than ICS, especially for complex pathogens with thousands of proteins such as malaria, which can be performed similarly in disparate hosts. Furthermore, tetramer staining marks Ag-specific cells with limited information about cell functionality. Following ICS with GP_{33} peptide, only ~80% of P14 cells that are known to recognize the GP_{33} epitope of LCMV responded with IFN–γ production (Figures 2 and 6), suggesting that some memory cells within the host are not capable of performing effector functions. At least some of these cells are likely to be T_{DIM} cells, which are generated during the process of homeostatic memory cell turnover, and are incapable of IFN–γ production or release of cytotoxic granules (62). Because host protection is ultimately dependent upon the number of cells present that are capable of responding to invading microbes with effector functions, ICS may provide a more accurate measure of protection than tetramer staining, as the former detects only cells capable of executing effector functions, while the latter detects cells that may be non-functional.

How can ICS be tailored to provide a more accurate assessment of Ag-specific CD8 T cell responses? Reductions in bystander activation can be achieved by selecting the shortest possible length of incubation and by adding BFA at the beginning of stimulation. However, when inclusion of BFA at the onset of ICS is not feasible due to stimulation with whole pathogen or proteins that require processing, additional steps can be performed to reduce bystander

activation and provide a more accurate enumeration of bona fide Ag-specific CD8 T cells. The severity of bystander activation can be mitigated by allowing pathogen processing and presentation to proceed first in a pure culture of antigen-presenting cells prior to the concurrent addition of syngeneic T cells and BFA. If such a strategy is not feasible, blocking antibodies to cytokines detected in the supernatant could be added during incubation to reduce bystander activation and increase accuracy.

Taking these steps to ensure accurate detection of Ag-specific effector or memory CD8 T cells will likely aid in evaluation and design of vaccines for infections of global importance and for cancer immunotherapy.

Methods

Human subjects, PfSPZ vaccination, and collection of PBMCs

Human samples from malaria vaccinated or naïve subjects were obtained from the VRC 314 study, as described (63). Briefly, healthy US adult volunteers were vaccinated intravenously with the PfSPZ Vaccine (64). EDTA-anti-coagulated whole blood was collected prior to vaccination and after the final vaccination. PBMCs were isolated by density gradient centrifugation and cryopreserved in LNVP.

Mice, infections, and generation of memory CD8 T cells

Inbred female C57Bl/6 mice were purchased from the National Cancer Institute (Frederick, MD) and bred at the University of Iowa, and TCR Tg P14 and OT-I mice were bred at the University of Iowa. All mice were used at 6-10 weeks of age and housed at the University of Iowa at appropriate biosafety levels.

All LCMV Armstrong infections were performed intraperitoneally with $2x10^5$ plaque forming units (PFU) per mouse. All *Listeria monocytogenes* (LM) infections were performed intravenously (i.v.- retroorbital injection) with $1x10^7$ colony forming units (CFU) per mouse of attenuated (Att) LM expressing the OVA_{257} peptide and the full length B8R protein from Vaccinia virus (VacV) (LM-OVA/B8R). For infections with radiation attenuated *P. berghei* sporozoites (Pb-RAS), *P. berghei* ANKA clone 234 sporozoites were isolated from the salivary glands of *A. stephensi* mosquitos purchased from the insectary of New York University. Sporozoites were attenuated by radiation with 200 Gray (Gy) by cesium irradiation prior to i.v. (retroorbital) injection of $2x10^4$ sporozoites per mouse. In vitro infections were performed using VacV expressing the OVA_{257} peptide (VacV-OVA), the GP_{33} peptide (VacV–GP_{33}), or the full-length nuclear protein (NP) from LCMV (VacV-NP; obtained from Dr. Steven Varga, Department of Microbiology and Immunology, University of Iowa, Iowa City, Iowa), or LM-OVA/B8R expressing the full-length OVA peptide and B8R peptide derived from VacV (Obtained from JD Sauer, Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, Wisconsin).

1° memory P14 cells were generated by adoptively transferring $5x10^3$ P14 cells obtained from peripheral blood of naïve P14 mice (Thy1.1/1.1 or Thy1.1/1.2) into naïve C57Bl/6 recipients (Thy1.2/1.2) followed by infection with LCMV. 1° memory OT-I cells were generated by adoptively transferring $5x10^3$ OT-I cells obtained from peripheral blood of naïve OT-I mice (Thy1.1/1.1 or Thy1.1/1.2) into naïve C57Bl/6 recipients (Thy1.2/1.2) followed by infection with LM-OVA/B8R.

ICS and flow cytometry for human and mouse samples

For figure 1, $1.5x10^6$ PBMCs from PfSPZ immunized or non-immunized (naïve) subjects were incubated with $1.5x10^5$ PfSPZs for the durations indicated. BFA (GolgiPlug™, BD Biosciences cat# 555029) was added to the culture medium at the times indicated at 10 μg/mL.

For figure 8, post vaccination PBMCs were CFSE labeled and mixed at a 9:1 ratio with pre-immunization PBMCs, and a total of $1.5x10^6$ PBMCs were incubated with $1.5x10^5$ PfSPZs for the times indicated, and with addition of BFA for the last 4 hours of incubation.

After stimulation, cells were stained as previously described (65). Briefly, cells were stained for viability with Aqua Live-Dead dye (Invitrogen), surface stained with CCR7 (clone Ax680, NIH vaccine research center), CD3 (clone SP34.2, BD), TCR-γδ (clone B1, BD), CD4 (clone OKT4, BioLegend), CD8 (clone RPA-T8, BioLegend), CD45RA (clone MEM-56, Invitrogen), and stained intracellularly for IFN–γ (clone4S.B3, BioLegend), IL-2 (clone MQ1-17H12, BioLegend), and TNF-α (clone Mab11, BioLegend). Flow cytometry data was acquired using a modified LSR-II (BD) and analyzed using FlowJo v9.9.6 (Tree Star Inc., Ashland, OR).

For human PBMCs examined in supplementary figures 3 and 7, LRS cones from a Trima Accel automated blood collection system (Terumo BCT, Lakewood, CO) were used to remove PBMCs, and the LRS cones were provided to investigators at the University of Iowa by the DeGowin blood center. PBMCs from cones were flushed by washing with complete RPMI followed by red blood cell lysis with ACK lysis buffer. PBMCs were then washed three times with complete RPMI and filtered through a 70 micron cell strainer before being resuspended in freezing media (90% Fetal Bovine Serum and 10%DMSO) and storage at -80°C. Cells were revived from frozen stocks by being thawed in a water bath followed by suspension in warmed complete media. Cells were then washed 3 times in warmed media and strained through a 70 micron cell strainer before $2x10^6$ cells were plated and incubated. For supplementary figure 3, cells were incubated with or without 100ng/mL human rIL-12 (BD Pharmingen) and IL-18 (Medical and Biological laboratories) for a total of 8 hours with BFA present the entire incubation

(0+8) or for the final hour (7+1), or for a total of 20 hours with BFA present the entire incubation (0+20) or for the final four hours (16+4). For supplementary figure 7, cells were incubated with or without 200nM concentrations of peptide pools consisting of CMV pp50 peptide (VTEHDTLLY- presented by HLA-A*0101 allele), CMV pp65 peptide (NLVPMVATV- presented by HLA-A*0201 allele) and EBV BMLF-1 peptide (GLCTLVAMD- presented by HLA-A*0201 allele) (all purchased from iba lifesciences), and with or without 0.6 μg/mL αIFN-γ, 0.6 μg/mL αTNF-α, 9 μg/mL αIL-12, and 9 μg/mL αIL-18 (all from R&D systems) for a total of 20 hours with BFA present the entire incubation (0+20) or for the final four hours (16+4). Cells were stained for surface expression of CD45RA (clone HI100, BioLegend), CD4 (clone A161A1, BioLegend), CD8 (clone HIT8a, BioLegend), and CD3 (clone HIT3a, BioLegend), and intracellular expression of IFN–γ (clone 4S.B3, BioLegend).

For mouse samples, spleens were collected and tissue was processed into single-cell suspension. Unless otherwise stated (Supplementary Figure 1), $2x10^6$ splenocytes were incubated with 200nM concentrations of GP_{33}, NP_{396}, or $GAP50_{40}$ peptide. Unless otherwise stated (Figures 4 and 5 and supplementary figures 2, 5, and 6), samples were incubated for a total of 8 hours with BFA present for the entire incubation (0+8) or for the final hour of incubation (7+1). In figure 4, cells were incubated for a total of 5, 8, 16, or 24 hours with BFA present for the entire incubation or for the final hour of incubation. In figure 5, cells were incubated for a total of 8 hours with BFA present for the entire incubation (0+8), for the final 6 hours of incubation (2+6), for the final four hours of incubation (4+4), or for the final hour of incubation (7+1). In supplemental figure 2, cells were incubated for a total of 8 hours with BFA present for the entire incubation (0+8), for the final four hours of incubation (4+4), or for the final hour of incubation (7+1), or for a total of 12 hours with BFA present for the entire incubation (0+12), for the final eight hours of incubation (4+8), for the final 4 hours of incubation (8+4), or for the final

hour of incubation (11+1). In supplemental figure 5, cells were incubated for a total of 12, 16, or 20 hours with BFA present for the final 4 hours of incubation. In supplemental figure 6, cells were incubated for a total of 12 hours with BFA present for the entire incubation (0+12), for the final eight hours of incubation (4+8), for the final 4 hours of incubation (8+4), or for the final hour of incubation (11+1).

In figure 3, P14 cells that were positively selected were added to splenocytes from an LCMV immune mouse prior to incubation. For positive selection, cells were stained with PE-anti-Thy1.1 antibodies (clone His51, eBioscience) and purified with anti-PE magnetic bead sorting using standard AutoMacs protocols.

In figure 6, splenocytes were incubated with or without 10ng/mL rIL-12 and IL-18 (R & D systems) in the presence or absence of 200nM GP_{33} or NP_{396} peptide. In supplementary figure 3, splenocytes were incubated with or without NP_{396} peptide or with or without rIL-12 and IL-18, IL-12 and TNF-α, or IL-12 and IL-15 (R & D systems).

In figure 7, splenocytes were incubated with or without 50μg/mL αIL-12 (C17.8), αIFN–γ (XM1.2), αTNF-α (XT22) (all produced in the Harty laboratory at the University of Iowa), or a mix of all anti-cytokines in the presence of 200nM NP_{396} peptide.

Following incubation, surface staining was conducted by incubating splenocytes with antibody cocktails for 20 minutes at 4° C. Endogenous (Thy1.1 neg) and P14 or OT-I (Thy1.1 pos) memory cells were distinguished from one another based upon surface staining with anti-CD8 (clone 53-6.7, eBioscience) and anti-Thy1.1 (clone His51, eBioscience). In figure 3, endogenous Ag-experienced CD8 T cells and P14 cells were detected based upon surface staining with anti-

573 Thy1.1 (clone His51, eBioscience), anti-CD8 (clone 53-6.7, eBioscience) and anti-CD11a (clone
574 M17/4, eBioscience) as previously described (49). Cells were then permeabilized and stained
575 intracellularly using anti-IFN–γ (clone XMG1.2, eBioscience). Flow cytometry data was acquired
576 using FACSCanto (BD Biosciences, San Jose, CA) and analyzed using FloJo software (Tree
577 Star Inc., Ashland, OR).
578
579 **ICS for mouse samples following stimulation with in vitro peptide-pulsed or whole**
580 **pathogens**
581 Splenocytes from a naïve C57BL/6 mouse were collected and processed into a single-cell
582 suspension. Cells were CFSE labeled by washing three times in PBS, incubating 10^7 cells/mL in
583 room temperature PBS for 15 minutes in the presence of 5mM CFSE, incubating on ice for 5
584 minutes with 1mL of fetal calf serum (FCS), and washing three times with RPMI containing 10%
585 FCS. Cells were re-suspended in RPMI containing 10% FCS.
586
587 Cells were then plated at $5x10^6$ cells per well, and RPMI containing 10% FCS, 200nM
588 concentrations of GP_{33} or NP_{396} peptide, or $5x10^6$ plaque forming units (PFUs) of VacV–GP_{33},
589 VacV-OVA, or VacV-NP were added to wells, and plates were incubated for 18 hours at 37° C.
590 Following incubation, cells were washed twice with RPMI containing 10% FCS and re-
591 suspended at $5x10^6$ cells per mL in RPMI containing 10% FCS.
592
593 $5x10^4$, $2.5x10^5$, $5x10^5$, or $2.5x10^6$ cells were plated with $2x10^6$ splenocytes from an LCMV
594 immune mouse, or from an LCMV immune mouse that received adoptive transfer of P14 cells
595 prior to infection. Cells were incubated for a total of 8 hours with BFA present during the entire
596 incubation (0+8), for the final six hours of incubation (2+6), for the final four hours of incubation
597 (4+4), or for the final hour of incubation (7+1).

598

599 Following incubation, cells were surface stained with anti-Thy1.1 (clone His51, eBioscience),
600 anti-CD8 (clone 53-6.7, eBioscience) and anti-CD11a (clone M17/4, eBioscience). Cells were
601 then permeabilized and stained intracellularly using anti-IFN–γ (clone XMG1.2, eBioscience).
602 data was acquired using FACSCanto (BD Biosciences, San Jose, CA) and analyzed using
603 FloJo software (Tree Star Inc., Ashland, OR).

604

605 In supplemental figure 5, splenocytes from an LCMV immune mouse containing P14 cells were
606 mixed with CFSE- labeled splenocytes from an LM-OVA/B8R immune mouse. $4x10^6$ cells
607 were then plated and incubated for 12, 16, or 20 hours with $5x10^6$ PFUs of VacV–GP_{33} or $1x10^7$
608 CFUs of LM-OVA/B8R and with or without 50μg/mL αIL-12, αIFN–γ, αTNF-α, or a mix of all
609 anti-cytokines, and with BFA present for the final four hours of incubation.

610

611 **Quantitative RT-PCR**

612 Spleens of mice containing memory P14 cells were collected and tissue was processed into
613 single-cell suspension. Cells were plated and incubated with 200nM NP_{396} peptide for a total of
614 8 hours with BFA present for the entire incubation (0+8) or for the final hour of incubation (7+1).
615 Cells were then surface stained for CD8 and Thy1.1 and purified with anti-PE magnetic bead
616 sorting (Miltenyi Biotec) using standard protocols. P14 cells were then sorted from purified cells
617 using a BD FACSAria II (BD Biosciences). Total RNA was reverse-transcribed using a
618 QuantiTech Reverse Transcription Kit (Qiagen), and cDNA was analyzed for expression of
619 *Il12rb2*, *Ifngr1*, *Ifngr2*, and *Tnfrsf1b* by quantitative PCR using SYBR Advantage qPCR premix
620 (Clontech) on an ABI 7300 Real Time PCR System (Applied Biosystems). Relative gene
621 expression levels in each sample were normalized to that of a housekeeping gene,
622 hypoxanthine phosphoribosyltransferase 1 (*Hprt1*).

Primers used were as follows:

Il12rb2: 5'- GTGTCTGCAGCCAACTCAAA and 3'- AGGCTGCCAGGTCACTAGAA

Ifngr1: 5'- GCTGGGTCCACTCTGCAAAT and 3'- GGCTTTGAGTAGCTTTCAGTTCAA

Ifngr2: 5'- GTGCTCCAAACACCGTGAAC and 3'- GCCACGTTGCCAGTAATGAG

Tnfrsf1b: 5'- TTGGGGCCGACTTGTTAAGG and 3'- TGGCTGTAAAGGTGGGATGG

Statistics

Statistical analyses were performed using GraphPad Prism software version 6 (GraphPad Software Inc.). Statistical comparisons of cytokine production by samples that were incubated in the presence of BFA for the entire incubation (0+8 or 0+20) compared to samples that were incubated with BFA for the final hour (7+1) or final four hours (16+4), or for mRNA expression of cytokine receptors for cells that were incubated in the presence of BFA for the entire incubation (0+8) compared to samples that were incubated with BFA for the final hour (7+1) were done using the paired 2-tailed student *t*-test, and a *p* value of less than 0.05 was considered significant. Statistical comparisons of cytokine production by human samples that were incubated in the presence of peptide pools and in the presence or absence of anti-cytokines and with BFA present for the entire incubation or the final four hours of incubation were done using a non-parametric ANOVA with repeated measures (Freedman's Test) with Dunn's post-hoc test for multiple comparisons with respect to 0+20 samples, and a *p* value of less than 0.05 was considered significant.

Study Approval

Human studies involving malaria vaccinated subjects were approved by the NIAID Institutional Review Board as described (63). All patients gave written informed consent. For blood donations collected from human patients at the University of Iowa, PBMCs were obtained from anonymous donors at the DeGowin Blood Center at the University of Iowa, and no identifying

information was collected from donors. Donors consented to allow blood cells not used for donation to be used for purposes of research. The consent process and documents for these donors have been approved by the IRB for the University of Iowa. All experiments involving animals were approved by the IACUC of the University of Iowa.

Author Contributions

M.D.M., I.J.J, A.S.I., Q.S., H.H.X., R.A.S., J.T.H., and V.P.B. designed the research studies; M.D.M., I.J.J., M.L., Q.S., and A.S.I. performed the research and analyzed the data; M.D.M., I.J.J., A.S.I., Q.S., R.A.S., J.T.H., and V.P.B. discussed the results and implications; and M.D.M., A.S.I., J.T.H., R.A.S., and V.P.B. wrote the paper.

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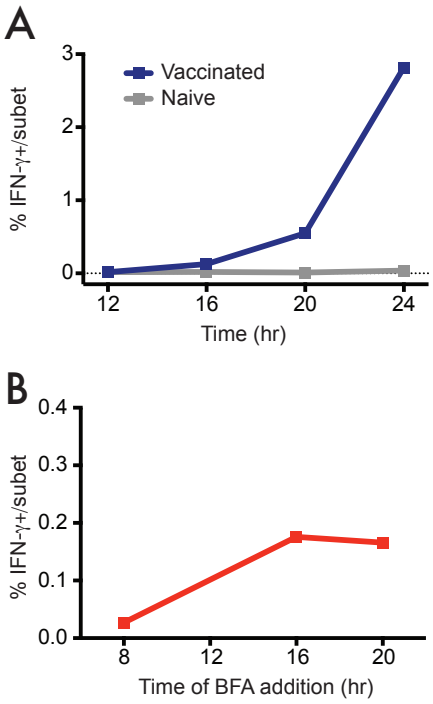
Figure 1


A
Vaccinated
Naive
% IFN-γ+/subset
3
2
1
0
12
16
20
24
Time (hr)
B
% IFN-γ+/subset
0.4
0.3
0.2
0.1
0.0
8
12
16
20
Time of BFA addition (hr)

875 **Figure 1. Increased length of incubation and delayed addition of BFA lead to elevated**
876 **detection of IFN–γ producing human CD8 T cells following PfSPZ stimulation. (A)**
877 Percentage of CD8 T cells producing IFN–γ when PBMCs from a PfSPZ vaccinated or
878 unvaccinated (naïve) subject were stimulated with PfSPZ for 12, 16, 20, or 24 hours. **(B)**
879 Percentage of CD8 T cells producing IFN–γ when PBMCs from a PfSPZ vaccinated subject
880 were stimulated with PfSPZ for a total of 24 hours with BrefeldinA (BFA) addition occurring after
881 the first 8, 16, or 20 hours. n=1
882

Figure 2

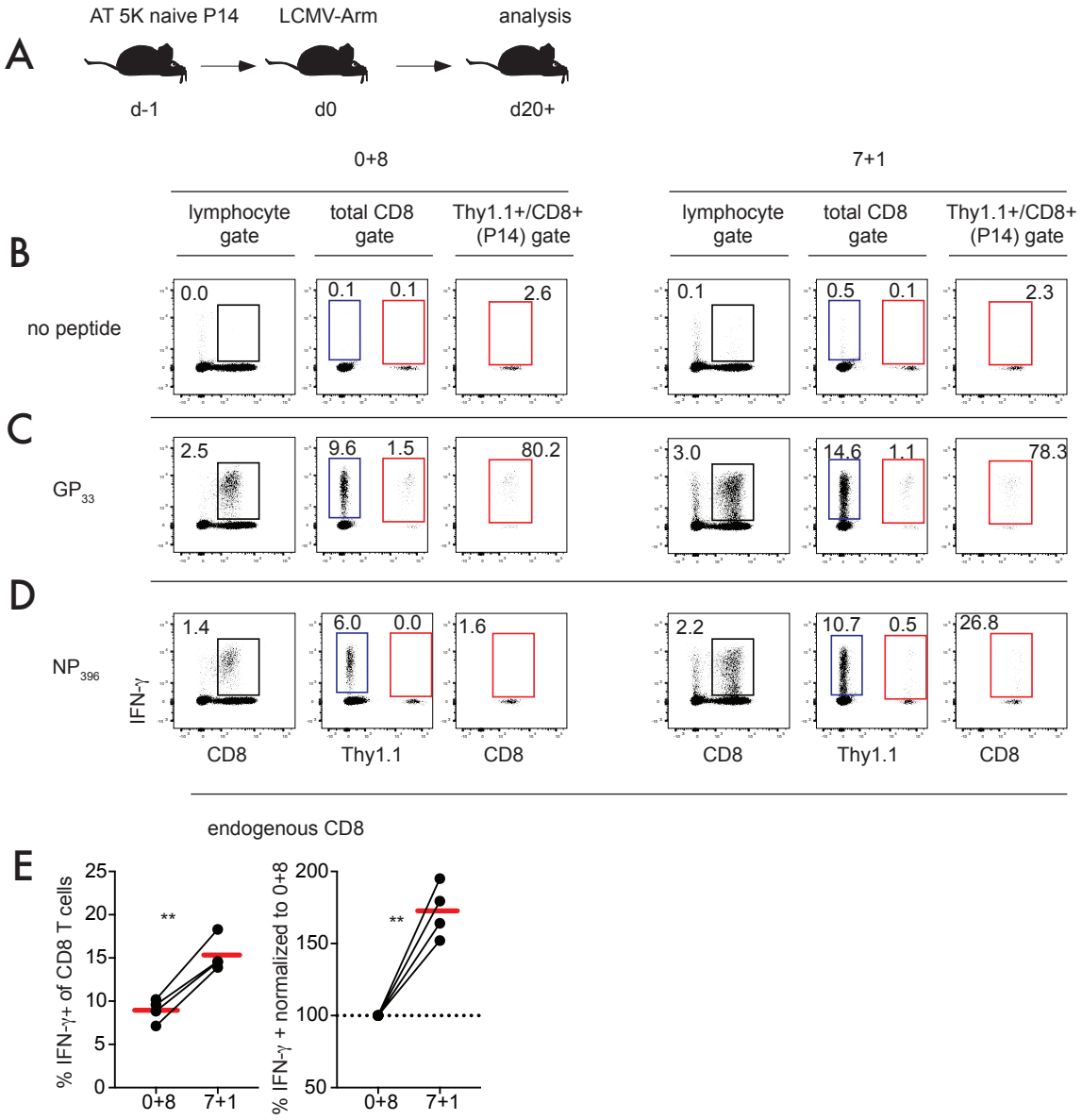

A
AT 5K naive P14
LCMV-Arm
analysis
d-1
d0
d20+
B
C
D
0+8
7+1
lymphocyte gate
total CD8 gate
Thy1.1+/CD8+ (P14) gate
no peptide
GP33
NP396
IFN-γ
CD8
Thy1.1
0.0
0.1
0.1
2.6
0.1
0.5
0.1
2.3
2.5
9.6
1.5
80.2
3.0
14.6
1.1
78.3
1.4
6.0
0.0
1.6
2.2
10.7
0.5
26.8
E
endogenous CD8
% IFN-γ+ of CD8 T cells
% IFN-γ + normalized to 0+8
**
0+8
7+1

Figure 2. Delayed addition of BFA leads to bystander activation of CD8 T cells. (A) Experimental design. Mice received adoptive transfer (AT) of naïve P14 cells and were infected with LCMV-Armstrong. Approximately three weeks after infection, splenocytes were harvested and ICS was conducted. **(B)** Representative dot plots of IFN–γ production following 8 hour incubation without peptide and with BFA present the entire incubation (0+8), or the final hour (7+1). Plots on the left are gated lymphocytes, plots in the middle are gated CD8 T cells (Thy1.1 neg= endogenous CD8 T cells, and Thy1.1 pos= P14 cells), and plots on the right are gated P14 cells. Numbers inside plots indicate the percentage of cells producing IFN–γ out of all gated cells. **(C)** Representative dot plots of IFN–γ production following 8 hour incubation with GP_{33} peptide. **(D)** Representative dot plots of IFN–γ production following 8 hour incubation with NP_{396} peptide. **(E)** Left- summary graphs of the percentage of endogenous CD8 T cells producing IFN–γ following stimulation with GP_{33} peptide out of all CD8 T cells with BFA present the entire incubation (0+8), or the final hour (7+1). Right- percentage of endogenous CD8 T cells producing IFN–γ when BFA was added the final hour of incubation normalized to the percentage when BFA was present the entire incubation (100%- dotted line). Representative data from greater than 3 independent experiments. n=4. Dots indicate individual mice. Solid red lines indicate the mean. $^{**}p<0.01$ as determined by paired student t test.

Figure 3

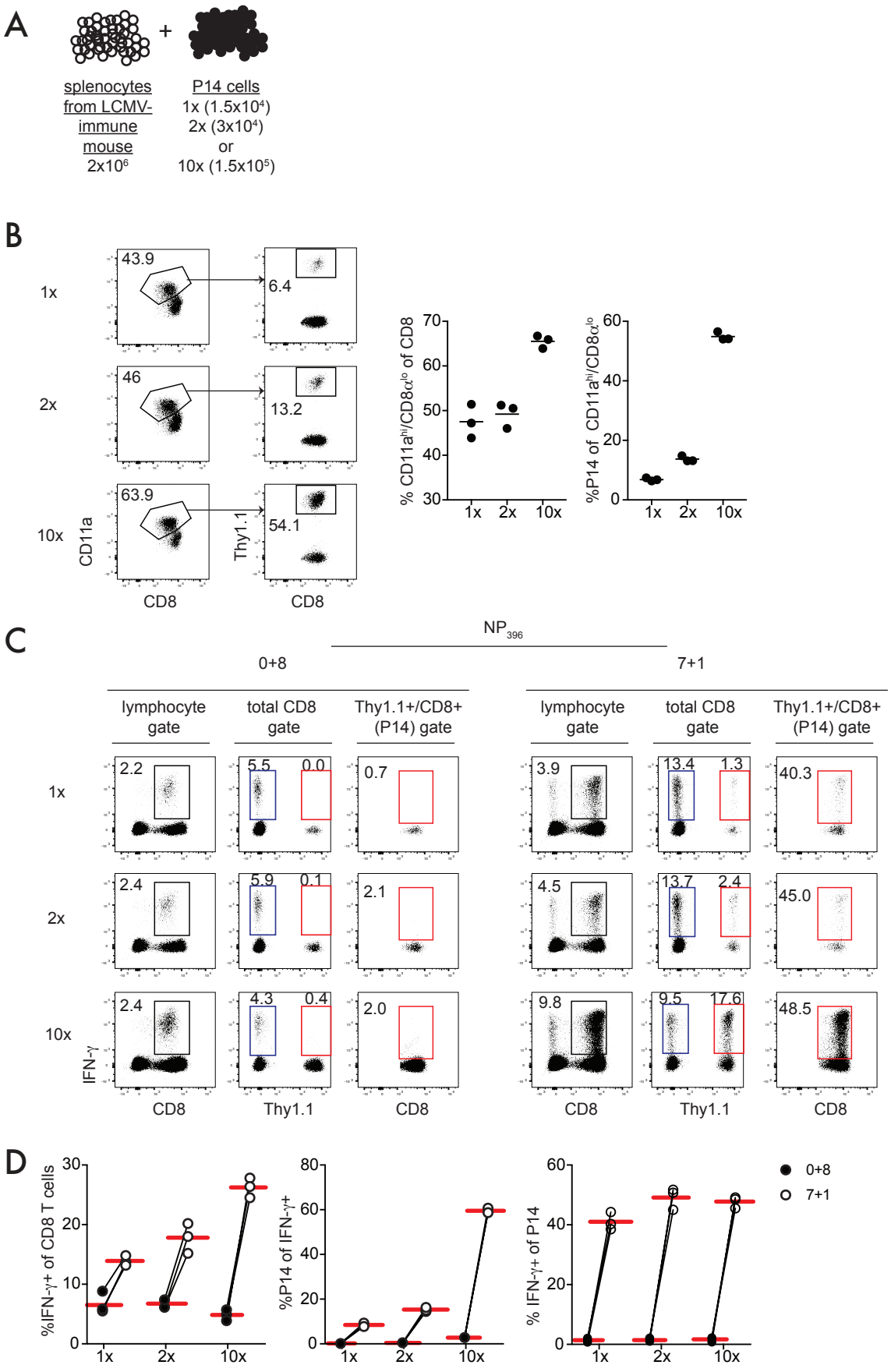

A
splenocytes from LCMV-immune mouse 2x10^6
P14 cells
1x (1.5x10^4)
2x (3x10^4)
or
10x (1.5x10^5)
B
1x
2x
10x
CD11a
CD8
Thy1.1
43.9
6.4
46
13.2
63.9
54.1
% CD11a^hi/CD8α^lo of CD8
%P14 of CD11a^hi/CD8α^lo
C
NP396
0+8
7+1
lymphocyte gate
total CD8 gate
Thy1.1+/CD8+ (P14) gate
IFN-γ
Thy1.1
2.2
5.5
0.0
0.7
3.9
13.4
1.3
40.3
2.4
5.9
0.1
2.1
4.5
13.7
2.4
45.0
2.4
4.3
0.4
2.0
9.8
9.5
17.6
48.5
D
%IFN-γ+ of CD8 T cells
%P14 of IFN-γ+
% IFN-γ+ of P14
0+8
7+1

Figure 3. Contribution of bystander responses to IFN–γ producing cells detected when addition of BFA is delayed is influenced by CD8 T cell pool composition. (A) Experimental design. Prior to 8 hour incubation with NP_{396} peptide, splenocytes from an LCMV-Armstrong immune mouse were mixed with different numbers of sorted memory P14 cells. **(B)** Left- representative dot plots of Ag-experienced (CD11ahi/CD8lo) CD8 T cells among all CD8 T cells (left plot) and percentage of P14 cells (Thy1.1 pos) among Ag-experienced CD8 T cells (right plot) after mixing. Middle-summary graph of the percentage of Ag-experienced CD8 T cells among all CD8 T cells after mixing. Right- summary graph of the percentage of memory P14 cells among Ag-experienced CD8 T cells after mixing. **(C)** Representative dot plots of IFN–γ production following 8 hour incubation with NP_{396} peptide and with BFA present the entire incubation (0+8), or the final hour (7+1). Plots on the left are gated lymphocytes, plots in the middle are gated CD8 T cells (Thy1.1 neg= endogenous CD8 T cells, and Thy1.1 pos= P14 cells), and plots on the right are gated P14 cells. Numbers inside plots indicate the percentage of cells producing IFN–γ out of all gated cells. **(D)** Left- summary graphs of the percentage of CD8 T cells producing IFN–γ out of all CD8 T cells with BFA present the entire incubation (0+8), or the final hour (7+1). Middle- summary graphs of the percentage of P14 cells producing IFN–γ out of all IFN–γ+ CD8 T cells with BFA present the entire incubation (0+8), or the final hour (7+1). Right- summary graphs of the percentage of gated P14 cells producing IFN–γ with BFA present the entire incubation (0+8), or the final hour (7+1). Representative data from 2 independent experiments. n=3. Dots indicate individual mice. Solid red lines indicate the mean.

Figure 4

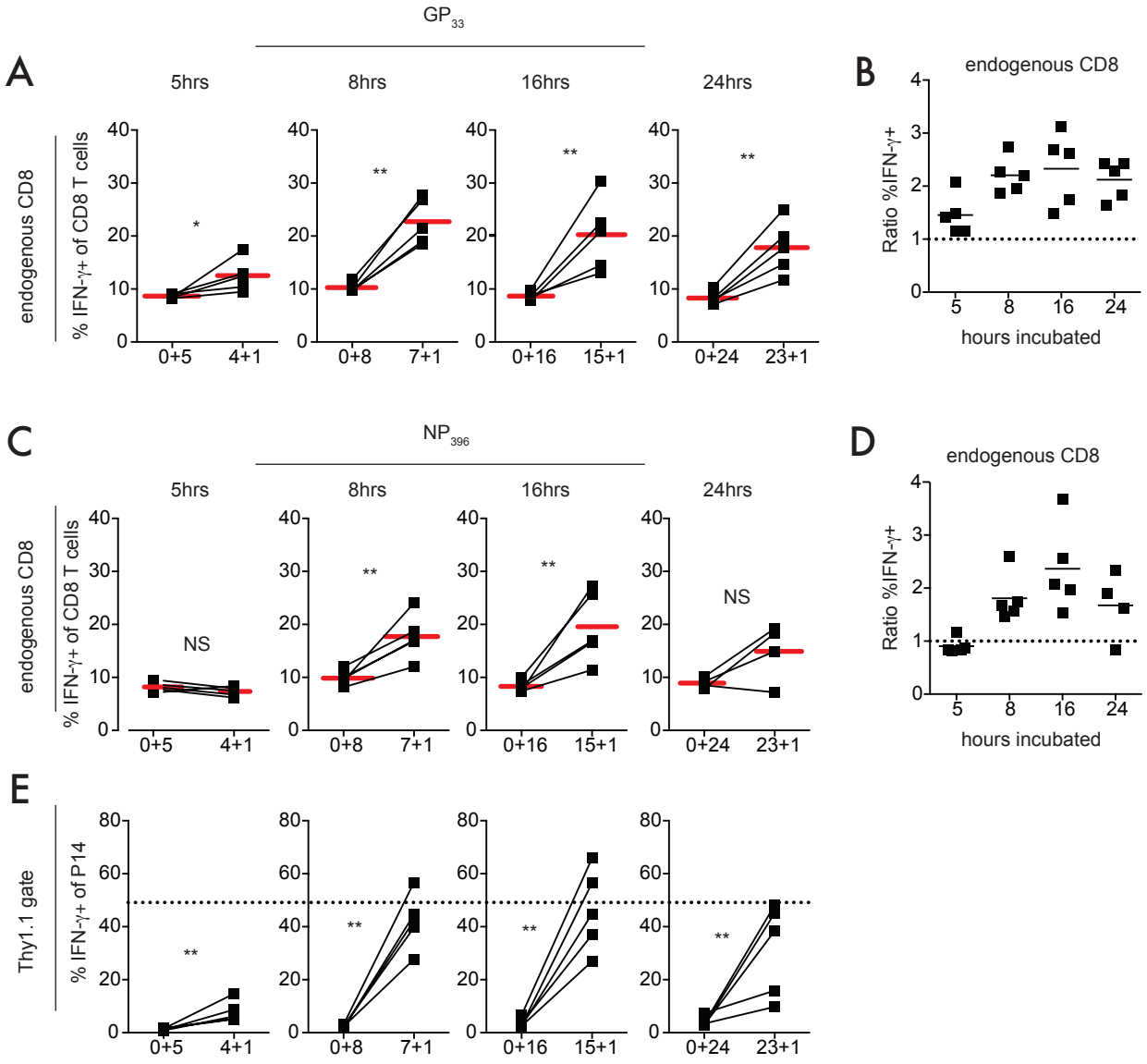

A
GP33
5hrs
8hrs
16hrs
24hrs
endogenous CD8
% IFN-γ+ of CD8 T cells
0+5
4+1
0+8
7+1
0+16
15+1
0+24
23+1
B
endogenous CD8
Ratio %IFN-γ+
hours incubated
C
NP396
NS
D
E
Thy1.1 gate
% IFN-γ+ of P14

Figure 4. Contribution of bystander responses to IFN–γ producing cells detected when BFA addition is delayed increases with extended length of stimulation but is seen following incubation times of 5 or more hours. Mice received adoptive transfer of naïve P14 cells and were infected with LCMV-Armstrong. ICS was conducted with GP_{33} **(A-B)** or NP_{396} **(C-D)** peptide approximately three weeks after infection. Total incubation times were 5, 8, 16, or 24 hours with BFA present the whole incubation or the final hour. **(A)** Summary graphs of the percentage of endogenous (Thy1.1 neg) CD8 T cells producing IFN–γ out of all endogenous CD8 T cells with BFA present the entire incubation, or the final hour. **(B)** Ratio of the percentage of endogenous CD8 T cells producing IFN–γ when BFA was present the final hour of incubation over the percentage of endogenous CD8 T cells producing IFN–γ when BFA was present the entire incubation. **(C)** Top- summary graphs of the percentage of endogenous (Thy1.1 neg) CD8 T cells producing IFN–γ out of all endogenous CD8 T cells with BFA present the entire incubation, or the final hour. Bottom- summary graphs of the percentage of P14 cells (Thy1.1 pos) producing IFN–γ out of all P14 cells with BFA present the entire incubation, or the final hour. **(D)** Ratio of the percentage of endogenous CD8 T cells producing IFN–γ when BFA was present the final hour of incubation over the percentage of endogenous CD8 T cells producing IFN–γ when BFA was present the entire incubation. Representative data from greater than 3 independent experiments. n=5. Dots indicate individual mice. Solid lines indicate the mean. NS=not significant, $^{*}p<0.05$, $^{**}p<0.01$ as determined by paired student t test.

Figure 5

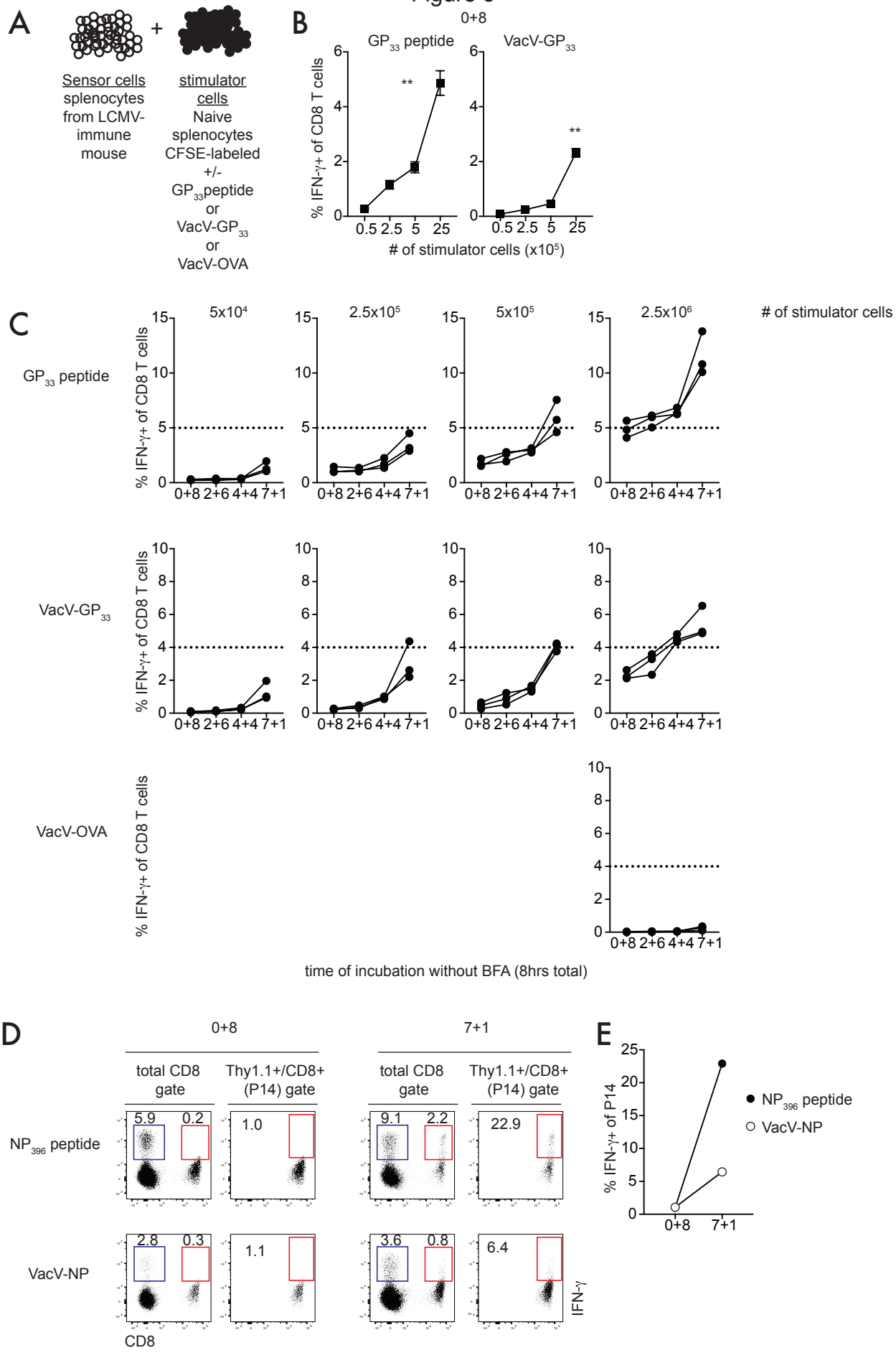

A
Sensor cells
splenocytes
from LCMV-
immune
mouse
stimulator
cells
Naive
splenocytes
CFSE-labeled
+/-
GP33peptide
or
VacV-GP33
or
VacV-OVA
B
0+8
GP33 peptide
VacV-GP33
% IFN-γ+ of CD8 T cells
# of stimulator cells (x10^5)
C
# of stimulator cells
5x10^4
2.5x10^5
5x10^5
2.5x10^6
GP33 peptide
VacV-GP33
VacV-OVA
% IFN-γ+ of CD8 T cells
0+8 2+6 4+4 7+1
time of incubation without BFA (8hrs total)
D
0+8
7+1
total CD8 gate
Thy1.1+/CD8+ (P14) gate
NP396 peptide
VacV-NP
5.9
0.2
1.0
9.1
2.2
22.9
2.8
0.3
1.1
3.6
0.8
6.4
CD8
IFN-γ
E
% IFN-γ+ of P14
NP396 peptide
VacV-NP

Figure 5. Delayed BFA addition leads to bystander activation of CD8 T cells following stimulation with pathogen-infected splenocytes. (A) Experimental design. Splenocytes from a naïve mouse (stimulator cells) were CFSE-labeled and either pulsed with GP_{33} peptide or infected with VacV–GP_{33} or VacV-OVA. Stimulator cells were mixed with splenocytes from an LCMV-Armstrong immune mouse (sensor cells) and incubated for 8 hours with BFA present for 8 (0+8), 6 (2+6), 4 (4+4), or 1 (7+1) hours. **(B)** CD8 T cells producing IFN–γ after 8 hour incubation with indicated numbers of GP_{33} peptide pulsed (left), or VacV–GP_{33} infected (right) stimulator cells with BFA present the entire incubation. **(C)** Top- CD8 T cells producing IFN–γ after incubation with indicated numbers of GP_{33} peptide- pulsed stimulator cells and with BFA the indicated times. Middle- CD8 T cells producing IFN–γ after incubation with indicated numbers of VacV–GP_{33} infected stimulator cells and with BFA the indicated times. Bottom- CD8 T cells producing IFN–γ after incubation with indicated numbers of VacV-OVA infected stimulator cells and with BFA the indicated times. **(D)** NP_{396} peptide-pulsed or VacV-NP infected stimulator cells were mixed with sensor cells from an LCMV-Armstrong immune mouse containing P14 cells. Representative dot plots of IFN–γ production by gated CD8 T cells (left plots- Thy1.1 neg= endogenous CD8 T cells, and Thy1.1 pos= P14 cells) or P14 cells (right plots). Numbers inside plots indicate the percentage of cells producing IFN–γ out of all gated cells. **(E)** Summary graphs of the percentage of P14 cells producing IFN–γ after stimulation with NP_{396} pulsed (black circles) or VacV-NP infected stimulator cells (white circles) with BFA present the entire incubation (0+8) or the final hour (7+1). Representative data from 3 independent experiments. n=3. Dots indicate individual mice. $^{**}p<0.01$ as determined by paired student t test.

Figure 6

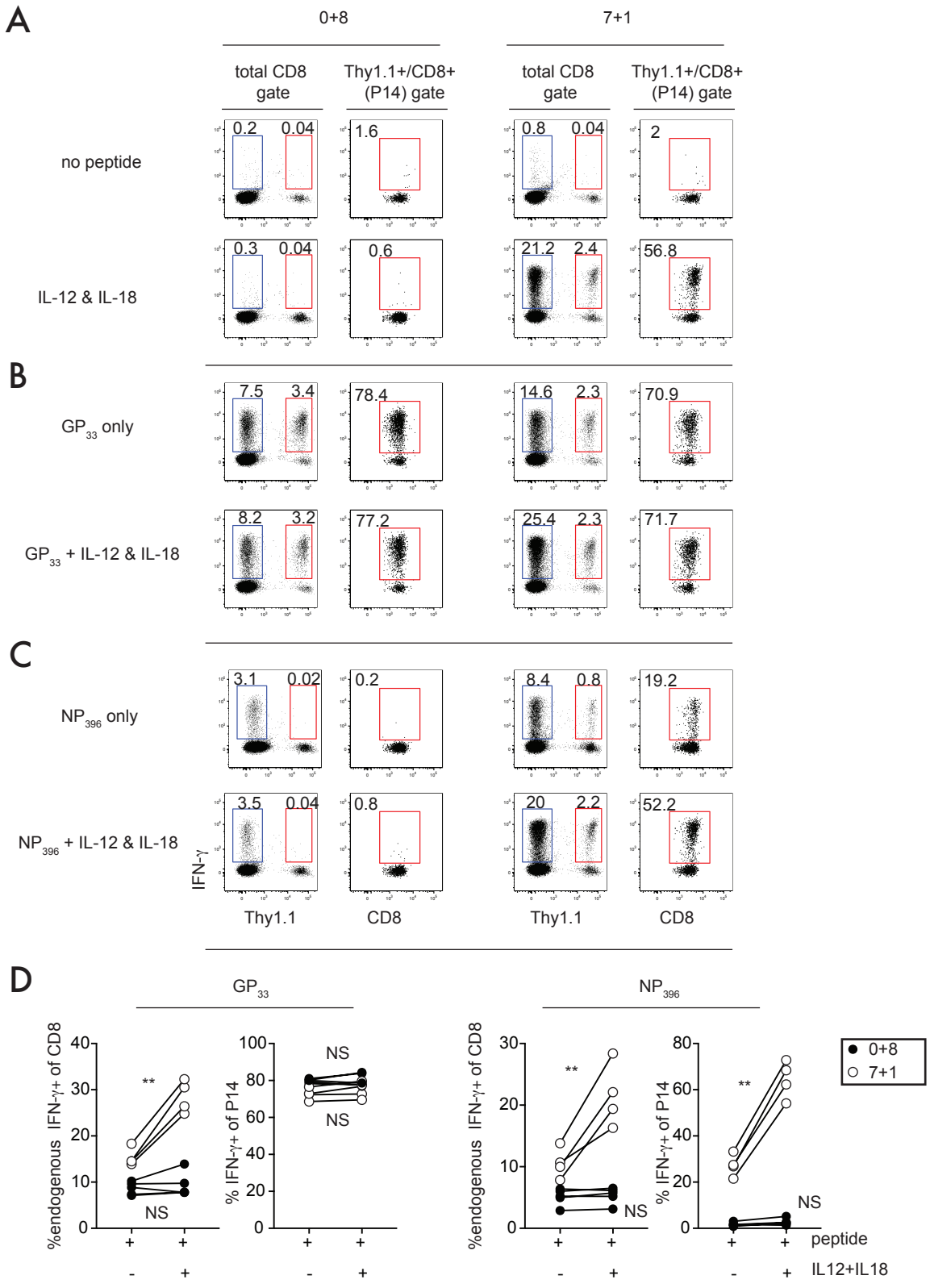

A
0+8
7+1
total CD8 gate
Thy1.1+/CD8+ (P14) gate
total CD8 gate
Thy1.1+/CD8+ (P14) gate
no peptide
IL-12 & IL-18
0.2
0.04
1.6
0.8
0.04
2
0.3
0.04
0.6
21.2
2.4
56.8
B
GP33 only
GP33 + IL-12 & IL-18
7.5
3.4
78.4
14.6
2.3
70.9
8.2
3.2
77.2
25.4
2.3
71.7
C
NP396 only
NP396 + IL-12 & IL-18
IFN-γ
3.1
0.02
0.2
8.4
0.8
19.2
3.5
0.04
0.8
20
2.2
52.2
Thy1.1
CD8
Thy1.1
CD8
D
GP33
NP396
%endogenous IFN-γ+ of CD8
% IFN-γ+ of P14
%endogenous IFN-γ+ of CD8
% IFN-γ+ of P14
**
NS
NS
NS
**
NS
**
NS
0+8
7+1
peptide
IL12+IL18

Figure 6. Inflammatory cytokines trigger bystander IFN–γ production by CD8 T cells when addition of BFA is delayed. Mice received adoptive transfer of naïve P14 cells and were infected with LCMV-Armstrong. ICS was conducted approximately three weeks after infection. **(A)** Representative dot plots of IFN–γ production following 8 hour incubation without peptide (top panels) or with IL-12 and IL-18 (bottom panels) and with BFA present the entire incubation (0+8), or the final hour (7+1). Plots on the left are gated CD8 T cells (Thy1.1 neg= endogenous CD8 T cells, and Thy1.1 pos= P14 cells), and plots on the right are gated P14 cells. Numbers inside plots indicate the percentage of gated cells producing IFN–γ. **(B)** Representative dot plots of IFN–γ production following 8 hour incubation with GP_{33} peptide (top panels) or with GP_{33} peptide and IL-12 and IL-18 (bottom panels). **(C)** Representative dot plots of IFN–γ production following 8 hour incubation with NP_{396} peptide (top panels) or with NP_{396} peptide and IL-12 and IL-18 (bottom panels). **(D)** Left- summary graphs of the percentage of endogenous CD8 T cells (left panel) and P14 cells (right panel) producing IFN–γ after incubation with GP_{33} peptide alone or with GP_{33} peptide and IL-12 and IL-18 with BFA present the entire incubation (black circles) or the final hour (white circles). Right- summary graphs of the percentage of endogenous CD8 T cells (left panel) and P14 cells (right panel) producing IFN–γ after incubation with NP_{396} peptide alone or with NP_{396} peptide and IL-12 and IL-18 with BFA present the entire incubation (black circles) or the final hour (white circles). Representative data from 2 independent experiments. n=4. Dots indicate individual mice. NS= not significant, $^{**}p<0.01$ as determined by paired student t test.

Figure 7

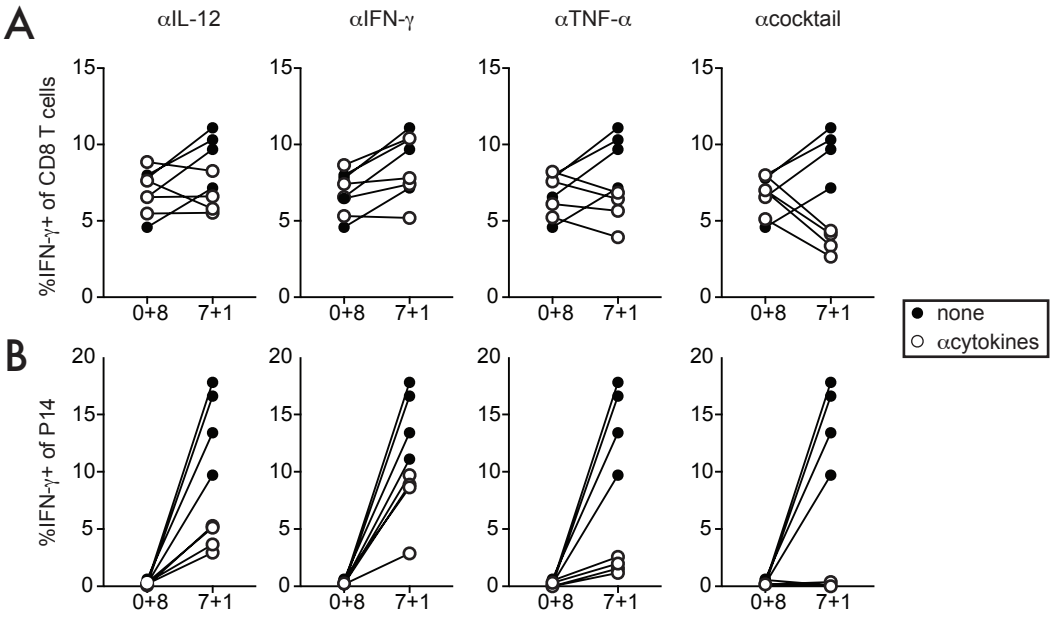

A
αIL-12
αIFN-γ
αTNF-α
αcocktail
%IFN-γ+ of CD8 T cells
0
5
10
15
0+8
7+1
none
αcytokines
B
%IFN-γ+ of P14
0
5
10
15
20
0+8
7+1

Figure 7. Blocking inflammatory cytokines reduces detection of bystander activated cells when addition of BFA is delayed. Mice received adoptive transfer of naïve P14 cells and were infected with LCMV-Armstrong. ICS was conducted approximately three weeks after infection. **(A)** Summary graphs of the percentage of endogenous CD8 T cells producing IFN–γ after incubation with NP_{396} peptide (black circles) or with NP_{396} peptide and 50μg αIL12, αIFN–γ, αTNF-α, or a mix of all cytokines (white circles) with BFA present the entire incubation (0+8) or the final hour (7+1). **(B)** Summary graphs of the percentage of P14 cells producing IFN–γ after incubation with NP_{396} peptide (black circles) or with NP_{396} peptide and 50μg αIL-12, αIFN–γ, αTNF-α, or a mix of all cytokines (white circles) with BFA present the entire incubation (0+8) or the final hour (7+1). Representative data from 2 independent experiments. n=4.

Figure 8

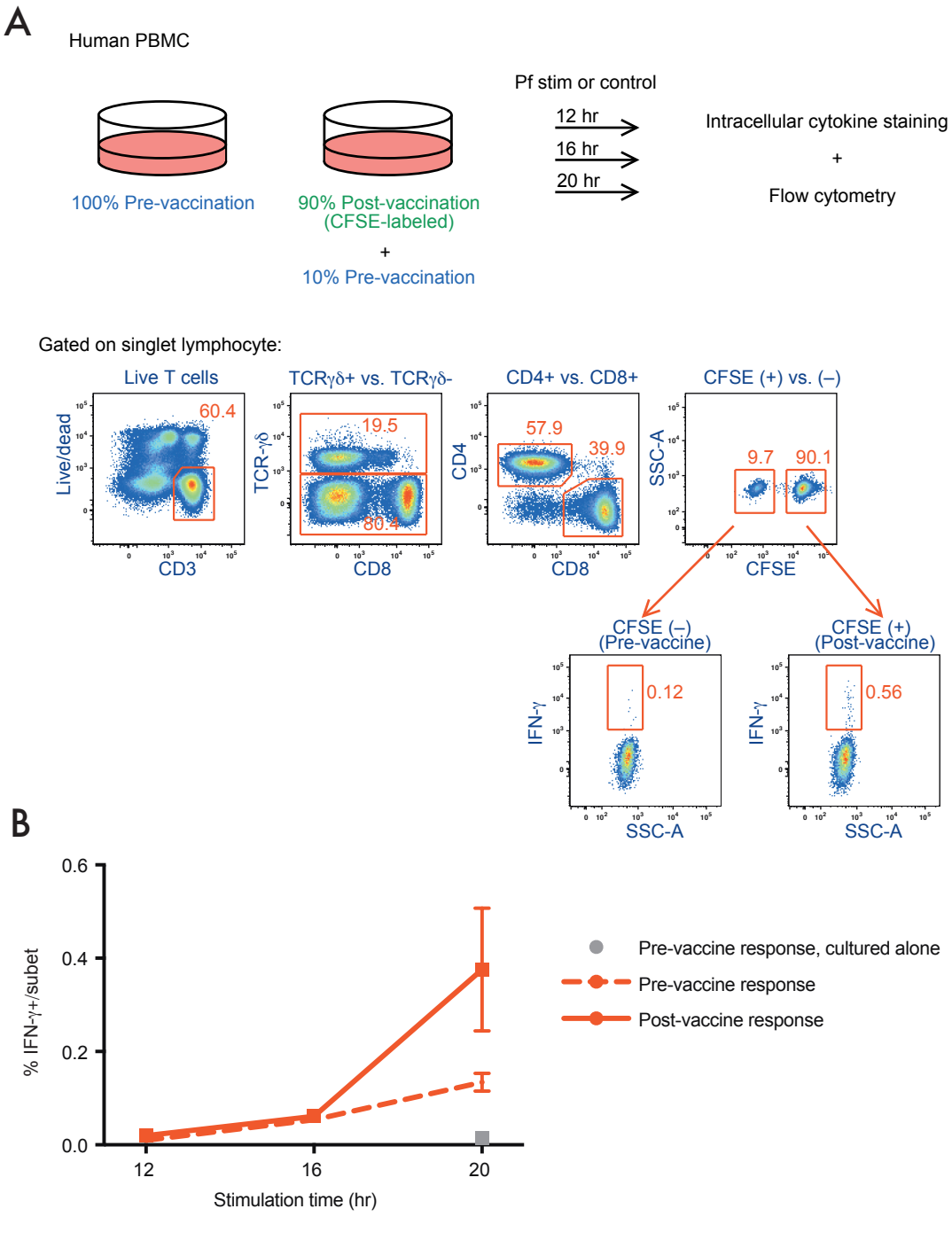

A
Human PBMC
100% Pre-vaccination
90% Post-vaccination
(CFSE-labeled)
+
10% Pre-vaccination
Pf stim or control
12 hr
16 hr
20 hr
Intracellular cytokine staining
+
Flow cytometry
Gated on singlet lymphocyte:
Live T cells
TCRγδ+ vs. TCRγδ-
CD4+ vs. CD8+
CFSE (+) vs. (–)
Live/dead
CD3
60.4
TCR-γδ
CD8
19.5
80.4
CD4
CD8
57.9
39.9
SSC-A
CFSE
9.7
90.1
CFSE (–)
(Pre-vaccine)
CFSE (+)
(Post-vaccine)
IFN-γ
SSC-A
0.12
0.56
B
% IFN-γ+/subet
0.0
0.2
0.4
0.6
12
16
20
Stimulation time (hr)
Pre-vaccine response, cultured alone
Pre-vaccine response
Post-vaccine response

Figure 8. Delayed addition of BFA leads to bystander activation of human CD8 T cells following stimulation with PfSPZ. PBMCs from PfSPZ vaccinated subjects were CFSE-labeled and mixed with non-labeled PBMCs from the same subjects (to allow for detection of bystander responses) that were collected prior to vaccination. Samples were then stimulated with PfSPZ for 12, 16, or 20 hours, and BFA was added for the last 4 hours of the incubation. PBMCs from subjects prior to vaccination were also stimulated in the absence of post-vaccination samples as a control. **(A)** Experimental design (top) and representative dot plot of the mix of pre-immunization (CFSE-neg) and post-immunization (CFSE-pos) PBMCs (bottom) following stimulation for 20 hours with PfSPZs. **(B)** Percentage of IFN-γ producing cells detected among pre-vaccine samples cultured alone (control), among pre-vaccine cells (bystander responses) cultured with post vaccine cells, and among post-vaccine cells cultured with pre-vaccine cells following stimulation. n=3. Data are mean +/- SEM.