

# The immune status of Kupffer cells profoundly influences their responses to infectious *Plasmodium berghei* sporozoites

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Multi-factorial immune mechanisms underlie protection induced with radiation-attenuated *Plasmodia* sporozoites ( $\gamma$ -spz). Spz pass through Kupffer cells (KC) before invading hepatocytes but the involvement of KC in protection is poorly understood. In this study we investigated whether  $\gamma$ -spz-immune KC respond to infectious spz in a manner that is distinct from the response of naive KC to infectious spz. KC were isolated from (1) naive, (2) spz-infected, (3)  $\gamma$ -spz-immune, and (4)  $\gamma$ -spz-immune-challenged C57BL/6 mice and examined for the expression of MHC class I and II, CD40 and CD80/CD86, IL-10 and IL-12 responses and antigen-presenting cell (APC) function. KC from  $\gamma$ -spz-immune-challenged mice up-regulated class I and costimulatory molecules and produced elevated IL-12p40, relative to naive KC. In contrast, KC from naive mice exposed to infectious spz down-modulated class I and IL-12p40 was undetectable. Accordingly, KC from spz-infected mice had reduced APC function, while KC from  $\gamma$ -spz-immune-challenged mice exhibited augmented APC activity. The nearly opposite responses are consistent with the fact that spz challenge of  $\gamma$ -spz-immune mice results in long-lasting sterile protection, while infection of naive mice always results in malaria.

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## Key words:

Kupffer cells  
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## Introduction

Sterile and protracted protection against malaria is inducible in humans [1] and rodents [2] by radiation-attenuated ( $\gamma$ ) *Plasmodia* sporozoites ( $\gamma$ -spz) and presently  $\gamma$ -spz are considered the only viable malaria vaccine [3]. Like infectious spz,  $\gamma$ -spz invade hepatocytes, however, they undergo aborted development and

form a repository of liver-stage Ag considered crucial for protection to exo-erythrocytic-stage parasites [4]. Protection is multi-factorial involving both innate and acquired immune responses [5]. We demonstrated that protection induced in C57BL/6 mice with *P. berghei*  $\gamma$ -spz is maintained by MHC class I-dependent [6], IFN- $\gamma$ -producing liver memory CD8 T cells [7]. There is a paucity of information, however, regarding the role of APC in the induction and maintenance of cells conferring protection and specifically the role of Kupffer cells (KC) remains unexplored.

KC reside in the lumen of hepatic sinusoids, which exposes them to Ag, hence singling KC as one of the key liver APC. Infectious *Plasmodia* spz pass through KC en route to hepatocytes [8, 9]. Upon invasion of or contact with KC, spz release the circumsporozoite (CS) protein, which binds to ribosomes and inhibits protein synthesis

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**Abbreviations:** IHMC: Intrahepatic mononuclear cells ·

$\gamma$ : Gamma irradiated · KC: Kupffer cells · MFI: Mean fluorescence intensity · P: *Plasmodium* · spz: Sporozoites

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[10], thereby disabling KC potential anti-parasitic function. However, KC can phagocytize spz [11] and interfere with the development of the exo-erythrocytic forms [12] presumably by the production of IL-6 [13]. *In vivo* depletion of KC prior to infection with *P. berghei* spz results in increased parasitemia [12]. Thus, interactions between KC and infectious spz vary; nonetheless, exposure of naive hosts to *Plasmodia* spz typically results in malaria.

It remains unknown whether  $\gamma$ -spz immunization changes the capacity of KC to respond to infectious spz in a manner distinguishable from the response of naive KC. We suggested [14] that the tolerogenic milieu in the liver [15, 16] favors *Plasmodia* spz colonization of and schizogony in hepatocytes; however, if  $\gamma$ -spz induce a local liver inflammation, the reversal of tolerance might facilitate induction of protective immunity. Therefore, we hypothesize that unlike naive KC,  $\gamma$ -spz-immune KC would respond to infectious spz in a manner characteristic of protective immunity, *i.e.* production of inflammatory cytokines, enhanced expression of MHC and costimulatory molecules and increased APC activity.

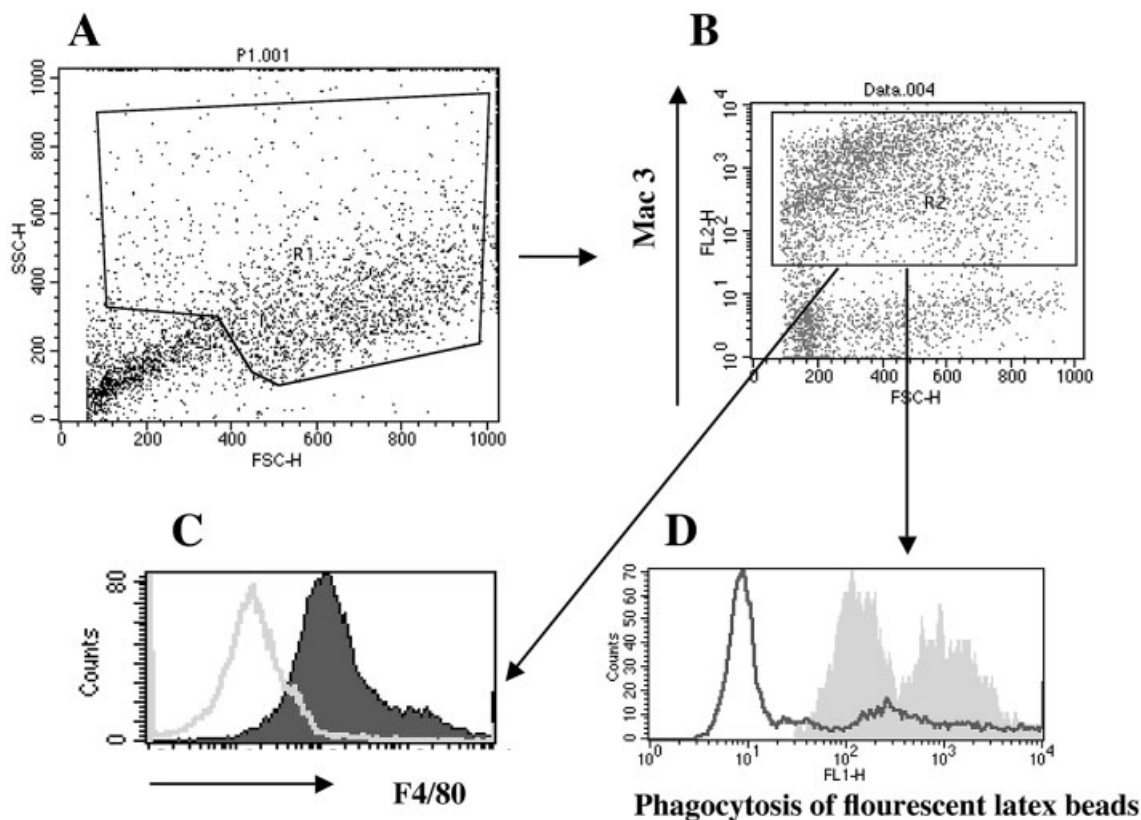
Using the *P. berghei*  $\gamma$ -spz protection model [6], here we demonstrate that after challenge, KC from  $\gamma$ -

spz-immune mice up-regulated MHC class I and costimulatory molecules and continued to produce IL-12. Naive KC responded in an opposite manner: the MHC class I was transiently down-regulated and IL-12 remained undetectable. The APC capacity of KC increased after challenge of  $\gamma$ -spz-immune mice but diminished after challenge of naive mice. Realizing the contributions of KC to protection would advance our understanding of anti-malaria protective mechanisms and hence, vaccine development.

## Results

### Identification of KC

To identify KC, we examined non-parenchymal liver cells for surface markers characteristic of KC and for phagocytosis. The forward and side scatter of Mac3<sup>+</sup> KC is shown (Fig. 1A). The gated Mac3<sup>+</sup> KC (Fig. 1B) expressed F4/80 (Fig. 1C) and internalized fluorescent beads at 37°C (filled) but not at 4°C (line contour) (Fig. 1D).



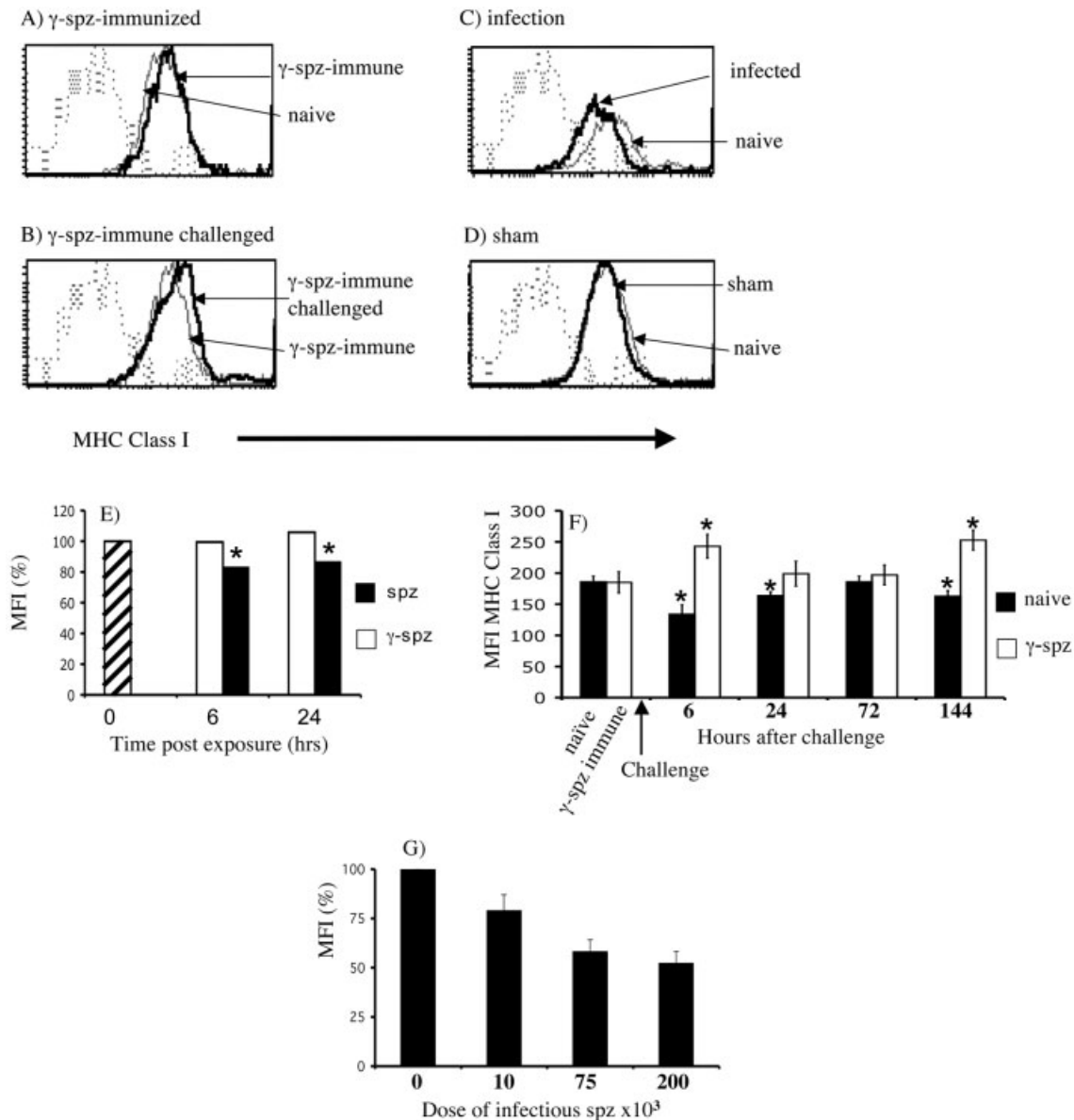
**Fig. 1.** Characterization of KC. KC were isolated from IHMC by magnetic depletion of T cells followed by positive selection of Mac3<sup>+</sup> cells. KC were analyzed by flow cytometry: (A) forward and side scatter; (B) gated Mac3<sup>+</sup> cells (C) expressed F4/80 (filled) and (D) internalized fluorescent latex beads at 37°C (filled) but not at 4°C (line).

## Infectious spz modulate MHC class I expression on KC

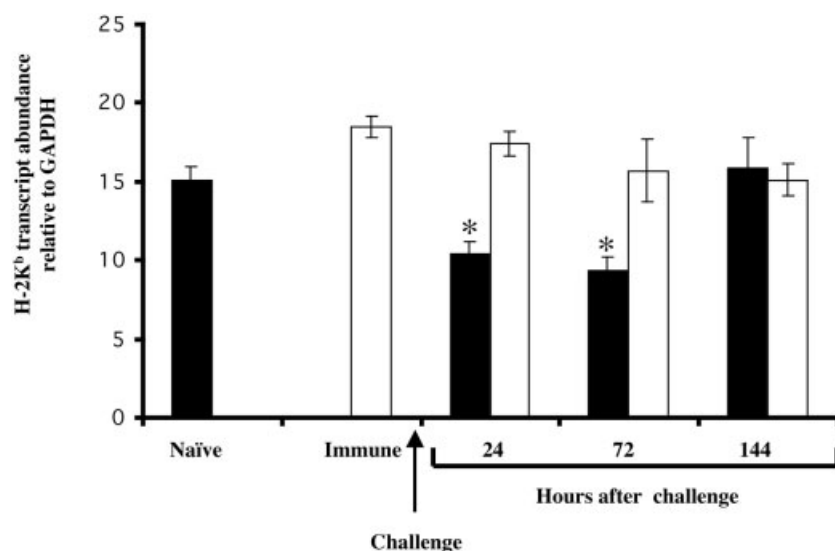
*P. berghei*  $\gamma$ -spz-induced protection in C57BL/6 mice is class I dependent [6], therefore, we asked whether modulations of MHC class I expression could be observed on KC under different conditions of exposure to spz. We compared KC from (1) naive, (2) naive spz-challenged, (3)  $\gamma$ -spz-immune and (4)  $\gamma$ -spz-immune-challenged mice. MHC class I remained constant after priming and boosting with  $\gamma$ -spz in comparison to naive

KC (Fig. 2A) or KC from mice immunized thrice with sham-dissected salivary gland debris (Fig. 2D). Mean fluorescence intensity (MFI) of MHC class I on  $\gamma$ -spz-immune KC (MFI = 190) vs. naive control KC (MFI = 195) is shown (Fig. 2F).

After challenge (10 K spz), KC from  $\gamma$ -spz-immune mice exhibited a significant increase of class I at 6 h (MFI = 250 vs. MFI = 190 for  $\gamma$ -spz-immune) (Fig. 2B, F) and at 144 h (MFI = 265) (Fig. 2F). The up-regulation was transient as it approached baseline at 24 and 72 h after challenge (Fig. 2F). By contrast, MHC class I was



**Fig. 2.** Spz-induced modulation of MHC class I expression on naive and  $\gamma$ -spz immune KC. Class I expression was determined by flow cytometry on KC from: (A–D) the indicated groups of mice either 72 h after the 3rd immunization with  $\gamma$ -spz or 6 h after challenge/infection; (E) naive mice (stripped), after a dose of 20 K infectious spz (black) or  $\gamma$ -spz (empty); (F) naive mice (black) or  $\gamma$ -spz-immune mice (empty) before and after spz challenge; (G) naive mice before and 24 h after infection with 10 K, 75 K and 200 K spz. Data in A–D are representative of results from three experiments ( $n = 2$ /group); dotted lines represent isotype controls. Composite data in E–G are from two to three experiments with two to three mice/group. MFI = 100% on naive KC. \* value  $p < 0.01$  indicates a significant difference between MHC class I on KC from experimental and naive mice.



**Fig. 3.** Differential expression of H-2K<sup>b</sup> heavy chain mRNA in KC from naive or  $\gamma$ -spz-immune mice before and after challenge. mRNA was isolated from KC from the groups of mice described in Fig. 2. H-2K<sup>b</sup> heavy chain transcripts were analyzed using semi-quantitative RT-PCR and samples were normalized against GAPDH gene. Naive KC were used as baseline. Bar graphs represent the mean  $\pm$  SD of triplicates from one of two representative experiments. \* value  $p < 0.01$  indicates a significant difference in comparison with naive KC H-2K<sup>b</sup> mRNA.

significantly decreased on naive KC at 6 (Fig. 2C) (MFI = 125 vs. naive controls MFI = 190), 24 (MFI = 165) and 144 h (MFI = 150) after the challenge (Fig. 2F). The diminished MHC class I returned to baseline at 72 h. Only infectious spz down-regulated class I on naive KC, as a single exposure to 10 K  $\gamma$ -spz (Fig. 2E) or sham-dissected debris (not shown) had no effect.

Inoculation with increasing doses (10 K – 200 K) of spz caused a gradual (20–45%) reduction in class I expression on KC (MFI = 100% for naive KC) (Fig. 2G). By contrast, priming with  $\gamma$ -spz (10 K – 75 K) did not affect class I expression (not shown). MHC class I also remained unaffected on hepatic DC, B and T cells at 6 and 24 h after the challenge of naive mice with 10 K spz (not shown).

### Infectious spz modulate MHC class I mRNA

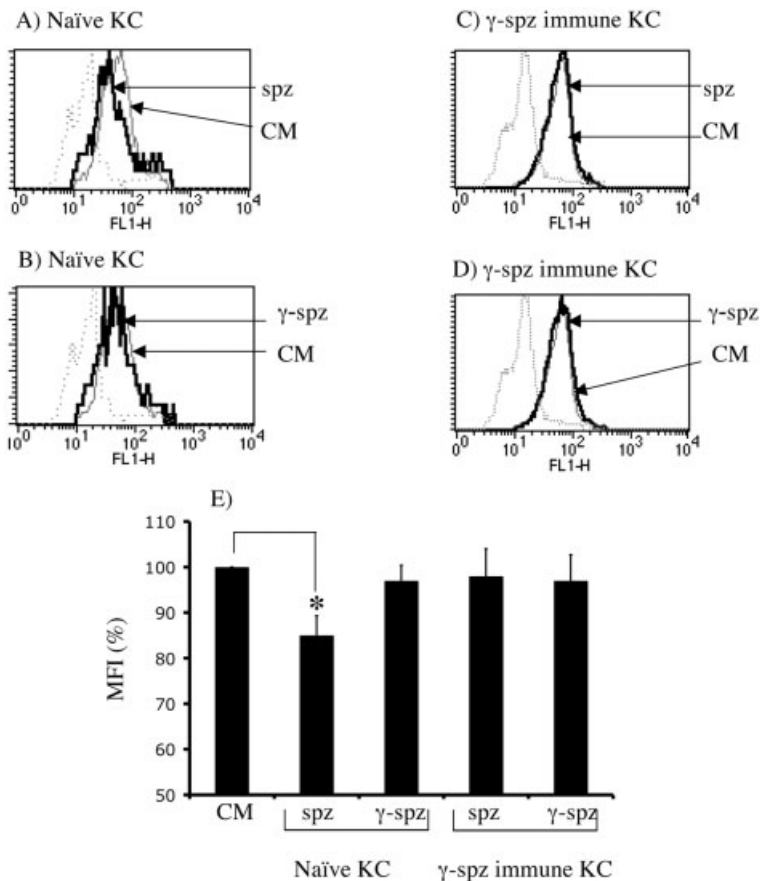
The down-regulation of class I implies spz-mediated interference with protein synthesis [10], assembly within the ER, or export to the surface of KC; however, changes could also occur at the nuclear level. Using RT-PCR, we compared levels of H-2K<sup>b</sup> heavy chain mRNA in KC isolated from naive and spz-infected mice as well as  $\gamma$ -spz-immune and  $\gamma$ -spz-immune-challenged mice. mRNA from naive-challenged KC decreased relative to mRNA from naive KC (Fig. 3). No changes in class I mRNA were observed in liver lymphocytes from the same group of mice (not shown). These data confirm our observations that spz down-regulate MHC class I and also show that events preceding the synthesis, assembly and transport of MHC class I are potential sites where the parasite controls immune responses of the host. We observed no increase in H-2K<sup>b</sup> mRNA in  $\gamma$ -spz-immune-challenged KC (Fig. 3).

### Spz mediate *in vitro* down-regulation of class I molecules on naive KC in the absence of other liver cells

It is unclear whether modulations of class I stem from a direct interaction between KC and spz or from cytokines released by other hepatic cells in response to spz. We cultured naive KC with infectious or  $\gamma$ -spz and analyzed their expression of class I (Fig. 4A, B). In comparison with control cultures, naive KC cultured with infectious but not with  $\gamma$ -spz showed  $\sim 15\%$  reduction in the expression of class I (MFI = 94 for KC with medium alone vs. MFI = 80 for KC plus infectious spz (Fig. 4A, E)). These results corroborate our observations *in vivo* and suggest that infectious spz interacted directly with KC without a contribution from other cells. By contrast, class I expression on  $\gamma$ -spz-immune KC remained refractory to modulation *in vitro* by either infectious or  $\gamma$ -spz (Fig. 4C, D, E). It appears that *in vivo* exposure to  $\gamma$ -spz obviated the down-regulatory effects of infectious spz observed with naive KC cells. The preemptive *in vivo* activity of  $\gamma$ -spz was likely caused by inflammatory cytokines produced by other liver cells responding to  $\gamma$ -spz.

### The expression of MHC class II on KC remains unchanged after exposure to spz

We asked whether spz-mediated modulation observed for class I was widespread, affecting other cell surface molecules. Because liver CD4 T cells are transiently activated during immunization with  $\gamma$ -spz [17], we considered, for example, that KC might up-regulate MHC class II. We analyzed class II expression at different times on KC from (1) naive, (2) naive-challenged, (3)  $\gamma$ -spz-immune and (4)  $\gamma$ -spz-immune-challenged mice. No changes were observed on KC after priming or boost



**Fig. 4.** *In vitro* spz-induced modulation of MHC class I expression on KC. KC isolated from (A and B) naive mice or (C and D)  $\gamma$ -spz-immune mice were cultured for 6 h with (A and C) infectious spz or (B and D)  $\gamma$ -spz. MHC class I expression was determined by flow cytometry: thick lines =  $\gamma$ -spz or infectious spz; thin lines = CM control; dotted lines = isotype controls. (E) Pooled results expressed as MFI are from two experiments ( $n = 3/\text{group}$ ). MFI of class I on KC cultured with CM is designated as 100%. \* value  $p < 0.01$  indicates a significant difference.

immunizations with  $\gamma$ -spz relative to naive KC (Fig. 5A). Challenge of  $\gamma$ -spz immune mice caused a small upward shift of class II on KC from 24 h (Fig. 5A) (MFI = 331 for  $\gamma$ -spz-immune vs. MFI = 424 for  $\gamma$ -spz-immune-challenged) to 144 h (not shown). The increase in class II, although not statistically significant, might have been sufficient to activate  $\gamma$ -spz-immune liver CD4 T cells. Class II remained unchanged on naive-challenged KC (Fig. 5A).

#### Expression of costimulatory molecules on KC is regulated differently during infection and immune challenge

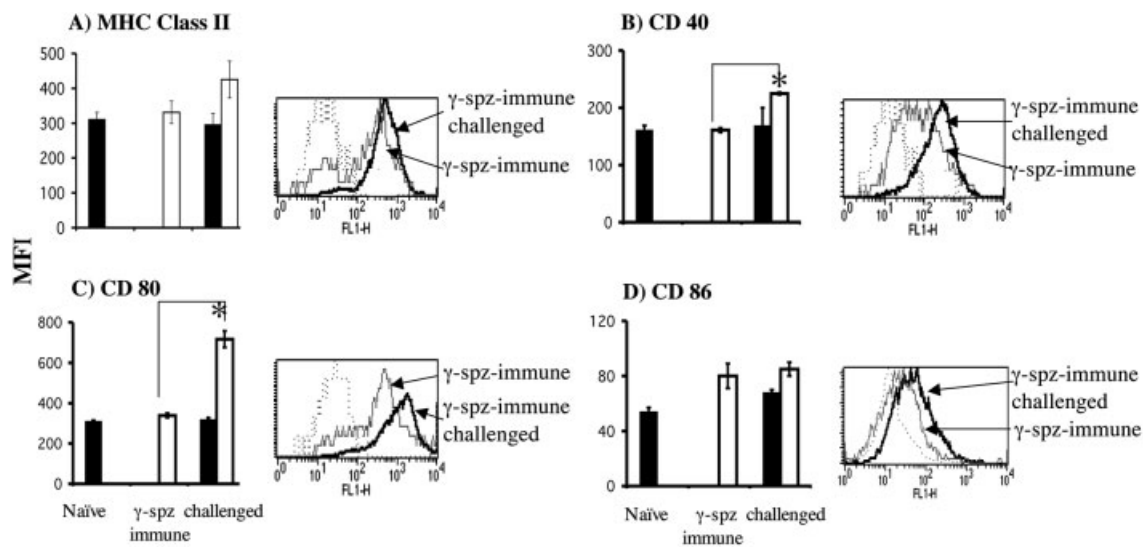
TCR-mediated activation of T cells also requires costimulation from CD40 and/or CD80/CD86 [18, 19]. If KC are involved in the activation of liver T cells, changes in the expression of these markers might occur during exposure to infectious and/or  $\gamma$ -spz. We measured CD40 and CD80/86 on KC after priming and boost immunizations with  $\gamma$ -spz and after spz challenge of naive and  $\gamma$ -spz-immune mice. CD40 expression on KC did not change after immunizations with  $\gamma$ -spz (MFI = 155 naive KC vs. MFI = 160  $\gamma$ -spz-immune KC) (Fig. 5B) but significantly increased (MFI = 225) (Fig. 5B) 24 h after the challenge and then returned to baseline at

144 h. Infection of naive mice caused no changes in the expression of CD40 on KC.

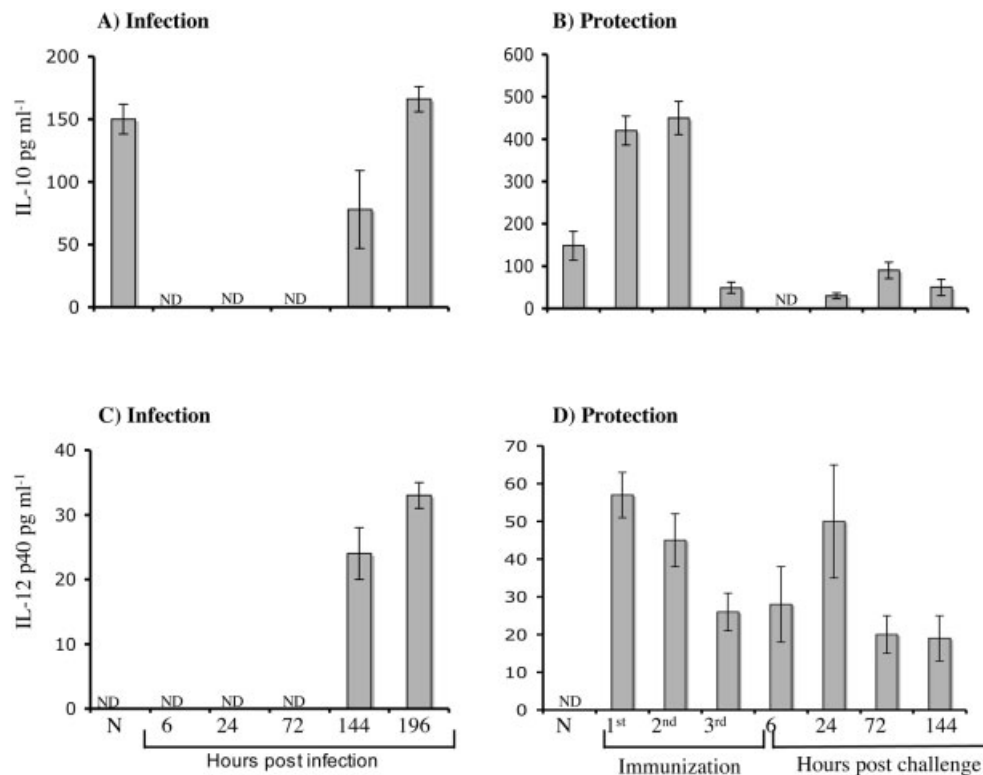
CD86 was mildly enhanced during immunizations with  $\gamma$ -spz (Fig. 5D). Challenge of naive or  $\gamma$ -spz-immune mice induced only a minor change in the expression of CD86 on KC (Fig. 5D and histogram). CD80 was not affected during immunizations with  $\gamma$ -spz (Fig. 5C) but it increased on KC 6 h (not shown) after challenge of  $\gamma$ -spz-immune mice and the shift became significant at 24 h (MFI = 760 for  $\gamma$ -spz-immune-challenged vs. MFI = 300 for  $\gamma$ -spz-immune) (Fig. 5C and histogram) and persisted for 72 h (not shown). Spz challenge or immunization with sham-dissected debris of naive mice caused but negligible changes in CD80 on KC (not shown).

#### IL-10 is reduced after exposure to infectious sporozoites

Having observed differential expression of surface markers on  $\gamma$ -spz-immune and naive KC in response to infectious spz, we asked if cytokine production by KC would be similarly affected. We analyzed IL-12 and IL-10 as they typically have opposing functions: inflammation and anti-inflammation.



**Fig. 5.** Expression of MHC class II and costimulatory molecules on naive and  $\gamma$ -spz immune KC. KC were isolated from either naive mice (filled columns) or  $\gamma$ -spz-immune mice 72 h after third immunization (open columns) or naive and  $\gamma$ -spz-immune mice 24 h after challenge. The levels of (A) class II, (B) CD40, (C) CD80 and (D) CD86 were determined by flow cytometry and the composite data expressed as MFI  $\pm$  SD are from three experiments ( $n = 2$ /group). Histogram inserts show representative data for KC from  $\gamma$ -spz-immune and  $\gamma$ -spz-immune-challenged mice. \* value  $p < 0.01$  indicates a significant difference between  $\gamma$ -spz-immune and  $\gamma$ -spz-immune-challenged KC.



**Fig. 6.** IL-10 and IL-12 production by naive and  $\gamma$ -spz-immune KC before and after exposure to infectious spz. KC were isolated from (A–D) naive mice; (B, D)  $\gamma$ -spz-immune mice 72 h after 1st, 2nd and 3rd immunizations; (A, C) naive-challenged mice and (B, D)  $\gamma$ -spz-immune-challenged mice at the indicated times after challenge. The cells were cultured for 24 h in CM and supernatants were analyzed for IL-10 and IL-12 p40 by ELISA, with a limit of detection for IL-10, 30 pg/ml, and for IL-12p40, 15 pg/ml. Values were calculated as the mean of triplicate wells  $\pm$  SD and are from one of three representative experiments,  $n = 2$  mice/group. ND = not detectable, N = naive.

**Table 1.** KC produce IL-10 and IL-12 in response to LPS stimulation<sup>a)</sup>

|                        | IL-10 pg/ml |          | IL-12 pg/ml |          |
|------------------------|-------------|----------|-------------|----------|
|                        | Medium      | LPS      | Medium      | LPS      |
| Naive                  | 140 ± 13    | 844 ± 37 | 0           | 31 ± 12  |
| spz-infected           | 0           | 174 ± 50 | 0           | 48 ± 4   |
| γ-spz-immune           | 49 ± 5      | 253 ± 13 | 26 ± 5      | 66 ± 5   |
| γ-spz-immune-challenge | 31 ± 3      | 176 ± 21 | 28 ± 7      | 163 ± 28 |

<sup>a)</sup> KC from naive, spz-infected, γ-spz-immune and γ-spz immune-challenged mice were cultured with or without LPS and analyzed by ELISA for IL-10 and IL-12p40 as described in the Materials and methods. The results represent the mean ± SD of composite data from three separate experiments.

Naive KC produced IL-10 *in vitro* (100 to 200 pg/ml) (Fig. 6A), as did KC from sham-immunized mice (not shown). γ-spz priming increased IL-10 threefold as compared to naive KC (Fig. 6B) but IL-10 decreased after 2<sup>nd</sup> boosting and challenge. IL-10 was undetectable in KC cultures from naive-challenge mice, although the onset of parasitemia caused IL-10 to return to the pre-immune level (Fig. 6A). The inability to detect IL-10 in KC cultures from naive-challenged mice might represent parasite-mediated cell death. However, caspase 3 determinations indicated that neither infectious nor γ-spz induced apoptosis beyond that observed in naive KC (not shown). We also tested IL-10 response in LPS-stimulated KC cultures. As shown in Table 1, LPS caused a sixfold increase of IL-10 in naive KC and although LPS-stimulated KC from spz-infected mice also produced IL-10, the level was approximately fivefold lower than in LPS-stimulated naive KC.

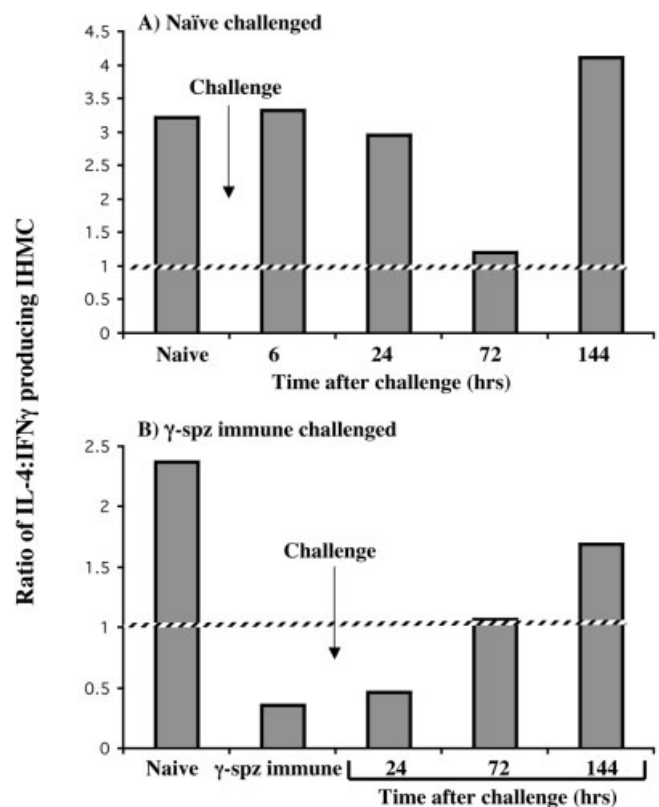
Our findings differ from those of Knolle et al.[20], who observed no IL-10 production by naive KC. It is possible that differences in the methods of KC isolation and IL-10 determination underlie these disparities. For example, use of Mac 3 mAb to enrich KC may have provided sufficient activation signal to induce low levels of IL-10.

### γ-spz but not infectious spz induce IL-12

KC isolated from naive and naive-challenged mice did not secrete IL-12p40 until the onset of blood-stage infection (144 h) (Fig. 6C), suggesting that spz did not perturb the state of tolerance in the liver. Injection of sham-dissected debris did not induce detectable IL-12p40 (not shown). LPS-stimulated KC from naive and spz-exposed mice produced measurable IL-12p40 (Table 1).

By contrast, starting 6 h after γ-spz priming, KC produced sustained IL-12p40 (Fig. 6D). These results suggest that γ-spz contributed to a switch from a state of tolerance to a local protracted inflammation, thus supporting the role of inflammation in protective

immunity [21]. KC from γ-spz-immune-challenged mice continued to produce IL-12, however, the levels fluctuated and were generally lower than those produced by KC from γ-spz primed and boosted mice (Fig. 6D).



**Fig. 7.** IL-4 prevails at pre-immunity but IFN-γ dominates during protection. IHMC were isolated from (A) naive mice and at the indicated times naive-challenged mice; (B) naive, γ-spz-immune (72 h after the third immunization) or γ-spz-immune-challenged mice. Ex vivo IL-4 and IFN-γ responses were determined by ELISPOT and the results are expressed as a ratio of IL-4:IFN-γ-producing cells. The number of cytokine-producing cells/10<sup>6</sup> IHMC in naive mice was: (A) 170 for IL-4 and 50 for IFN-γ; (B) 156 for IL-4 and 65 for IFN-γ. The data are representative of three or more experiments, *n* = 5 mice/group.

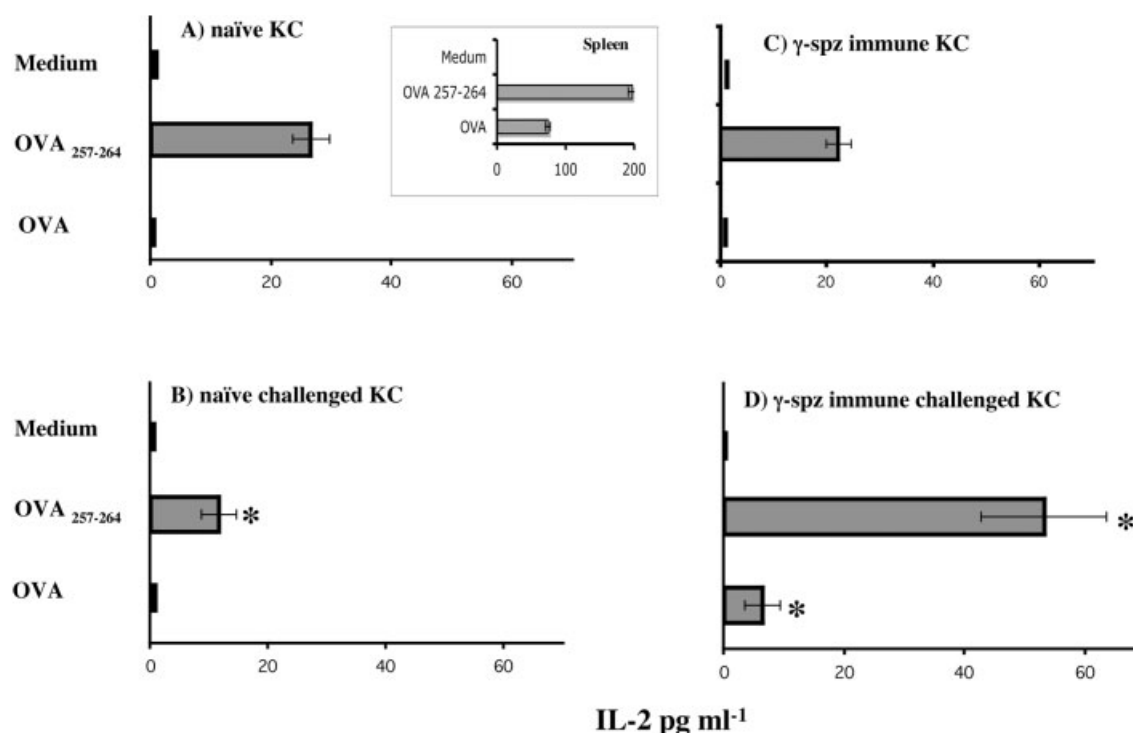
## IL-4 prevails in non-immune liver but IFN- $\gamma$ dominates during protection

We asked whether IL-4 and IFN- $\gamma$  responses of intrahepatic mononuclear cells (IHMC) would mirror cytokine production by KC. IHMC were isolated at different times after exposure of naive as well as  $\gamma$ -spz-immune mice to infectious spz and IL-4 and IFN- $\gamma$  responses were determined *ex vivo*. Naive IHMC produced IL-4, while IFN- $\gamma$  response was negligible (IL-4:IFN- $\gamma$ -producing cells ratio = 3) (Fig. 7A). The high IL-4:IFN- $\gamma$  ratio was maintained after exposure of naive mice to infectious spz (Fig. 7A). It is possible that parasite-induced IL-4 controls inflammation in the liver by down-regulating IL-12R [22].

In contrast, immunization with  $\gamma$ -spz increased IFN- $\gamma$  responses and although IL-4 was maintained, the ratio of IL-4:IFN- $\gamma$ -producing cells decreased to 0.5 (Fig. 7B). This switch is consistent with the observation that KC produced IL-12 immediately after  $\gamma$ -spz priming. Although IFN- $\gamma$  dominated the  $\gamma$ -spz-induced response, IL-4 was restored after challenge, with IL-4:IFN- $\gamma$  ratio approaching 1 at 72 h.

## Infectious *P. berghei* spz reduce the APC capacity of KC

KC present Ag to T cells but less efficiently than spleen- or bone marrow-derived macrophages [23]; however, their APC function during Plasmodia infection or protection remains unknown. We examined KC capacity to process and/or present OVA and the OVA peptide<sub>257–263</sub> (SIINFEKL) to the OVA<sub>257–263</sub>-specific T cell hybridoma, RF33:70 [24]. Naive KC presented SIINFEKL to the RF33:70 T cells (Fig. 8A) but the T cell response (IL-2) was moderate in comparison to cultures with splenic APC (insert Fig. 8A) and naive KC did not present OVA (Fig. 8A). KC from spz-infected mice presented SIINFEKL  $\sim$  50% less efficiently than naive KC (Fig. 8B). In contrast, KC from  $\gamma$ -spz immune-challenged mice manifested the most efficient peptide presentation (IL-2: 50 pg/ml) (Fig. 8D) and, albeit less efficiently, they also presented OVA (IL-2: 8 pg/ml).  $\gamma$ -spz-immune KC functioned similarly to naive KC (Fig. 8C). Thus, infectious spz limited the ability of naive KC to function as APC, whereas both presentation of SIINFEKL and processing of OVA were enhanced in  $\gamma$ -spz-immune-challenged KC.



**Fig. 8.** Infectious and  $\gamma$ -spz influence the APC capacity of KC. KC from the indicated groups (A–D) were pulsed for 1 h with OVA (22  $\mu$ M) or OVA<sub>257–264</sub> peptide (0.1  $\mu$ M) or medium. Ag-pulsed KC ( $1 \times 10^5$ ) were cocultured with SIINFEKL-specific T hybridoma cells ( $1 \times 10^5$ ) for 48 h and supernatants were analyzed for IL-2 (ELISA detection limit: 3 pg/ml). The insert shows splenic APC activity. The results, from one of three representative experiments ( $n = 3$  mice/group) show the mean of triplicate wells  $\pm$  SD. \*  $p < 0.01$  indicates a significant difference relative to presentation by naive KC.

## Discussion

We characterized immune responsiveness of KC in protection induced by *P. berghei*  $\gamma$ -spz. We provide evidence that immunization with  $\gamma$ -spz influenced reactivity of KC to infectious spz: MHC class I, CD40 and CD80 increased as did the APC capacity; IL-12 was induced and maintained, and IFN- $\gamma$  production by IHMC mirrored the KC response. Reactivity of naive KC to infectious spz contrasted sharply: MHC class I decreased both *in vivo* and *in vitro* as did APC function; IL-12 remained undetectable and IL-10 also decreased. We cannot conclude with certainty that  $\gamma$ -spz-induced responses of KC contribute to protective immunity; nonetheless, the polarized responses of naive versus  $\gamma$ -spz-immune KC that developed after challenge are in concordance with the observations that while naive hosts develop malaria,  $\gamma$ -spz-immune hosts resist spz challenge.

The anti-Plasmodia activity of KC has been previously reported [12]. Our study, however, is the first to demonstrate the influence of  $\gamma$ -spz on responses of KC to infectious spz. We maintain that  $\gamma$ -radiation altered the manner or the course of spz interaction with KC and possibly other liver cells. The effects of radiation might not be uniform; the less damaged  $\gamma$ -spz might enter KC by membrane:membrane interaction and localize in a vacuole distant from the phagosome [8], while extensively damaged  $\gamma$ -spz might be phagocytized [8]. The presence of CS protein-specific Ab [6] might enhance phagocytosis of  $\gamma$ -spz as well as infectious spz, thus potentially channeling spz to the phagosome/ER compartment for cross-presentation by the class I pathway [25].

A transient, albeit significant, increase of class I on  $\gamma$ -spz-immune KC shortly after challenge was likely caused by temporary local inflammation in the liver induced by  $\gamma$ -spz. Challenge of  $\gamma$ -spz-immune mice maintained IL-12 production by KC, while exposure of naive mice to infectious spz failed to induce IL-12 in KC and INF- $\gamma$  in IHMC. Inflammatory cytokines enhance proteasome activity and hence availability of peptides for export by class I [26]. The increased class I on  $\gamma$ -spz-immune KC might reflect a rapid/efficient export of recycling class I molecules because we observed no increase in K<sup>b</sup> heavy chain mRNA. The refractoriness of  $\gamma$ -spz-immune KC to up-regulate class I in response to infectious spz *in vitro* strongly suggests a significant *in vivo* contribution of  $\gamma$ -spz-induced inflammatory cytokines produced by other liver cells.

We propose that up-regulated class I molecules on KC trigger liver effector CD8 T cells to release INF- $\gamma$ , a cytokine necessary for anti-parasitic activity. The ratio of IL-4:INF- $\gamma$ -producing cells was also biased toward inflammation during protection, whereas IL-4 was the

dominant cytokine during parasitemia. Th1-type responses also control *Leishmania major* but Th2-type responses exacerbate the disease [27]. This is consistent with our observation [7] that IFN- $\gamma$ -producing liver CD8 T cells increase between 1 and 6 h after challenge of  $\gamma$ -spz-immune mice. Effector CD8 T cells also might require costimulation by CD40 and/or CD80, which increased on  $\gamma$ -spz-immune KC after challenge; moreover, they might rely on activated CD4 T cells, thus it is relevant that class II molecules on KC increased, albeit insignificantly, after challenge of  $\gamma$ -spz-immune mice.

The selective down-regulation of MHC class I on and the absence of early IL-12 production by KC from naive-challenged mice suggest that spz-induced inhibition targeted predominantly MHC class I and inflammatory cytokines, as reported in other systems [27]. MHC class I and inflammatory cytokines are *sine qua non* for the activation of Ag-specific effector CD8 T cells, which are not detected in spz-challenged naive mice [7]. Spz may have interacted directly or indirectly with KC. According to the shift in MHC class I expression, spz affected the majority of Mac3<sup>+</sup> KC, suggesting a rather extensive modulation, typically achievable by a soluble factor or possibly by CS protein, which binds to proteoglycans on the KC [28] and also inhibits protein synthesis [10]. Moreover, down-regulation of class I by spz *in vitro* appears to involve KC independent of other liver cells. If CS protein is the primary mediator, the transient down-regulation could be explained by the lack of CS protein synthesis once the spz enters hepatocytes [29]. Conversely, infectious spz transverse many hepatocytes [30] and it is possible that they also transverse many KC and temporarily reduce their APC capacity as we observed for KC from naive-challenged mice.

The requirement for IL-12 as a mediator of  $\gamma$ -spz-induced protection has been established in IL-12KO mice [21]. By contrast, IL-10 might limit inflammation in the vicinity of spz invasion. IL-10 inhibits some T cell responses to *P. falciparum* Ag [31], but IL-10 also accompanies protective immunity in malaria endemic areas [32]. Hence, it is possible that the inflammatory and regulatory cytokines are coordinately induced during protection against malaria.

In summary, we previously hypothesized [33] that the prevailing immuno-tolerant condition in the liver might favor the colonization of hepatocytes by Plasmodia spz. Here we demonstrate that infectious spz caused a reduction in the expression of and mRNA for MHC class I and did not induce naive KC to secrete IL-12, which likely promoted successful spz invasion of hepatocytes. The kinetics of MHC class I up-regulation on  $\gamma$ -spz-immune-challenged KC and production of IL-12 suggest a potential involvement of KC in protective immunity; however, the absence of increased class I on  $\gamma$ -spz-immune KC in response to infectious spz *in vitro*

suggests a contribution of inflammatory cytokines produced by other  $\gamma$ -spz-primed liver cells responding *in vivo* to spz. The data presented here are consistent with the role of CD8 T cells and inflammatory cytokines in  $\gamma$ -spz-induced protection. In contrast, down-regulated class I and a lack of IL-12 production by naive KC exposed to infectious spz partly explain the absence of CD8 T cells and hence clinical malaria.

## Materials and methods

### Mice

Six- to eight-week-old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were housed according to institutional guidelines. Research was conducted in compliance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals (1996). Procedures were approved by the IACIC and the AAALACI.

### Preparation of spz, immunization and challenge

*P. berghei* spz (ANKA strain), maintained by cyclical transmission in mice and *Anopheles stephensi*, were dissected from mosquitoes 21 days after blood meal and used either immediately or after attenuation with  $\gamma$ -radiation (15,000 rad) (Cesium-137 source Mark 1 series; JL Shepard & Associates, San Fernando, CA). Sham-dissected preparations obtained from non-infected mosquitoes were treated identically. Mice were primed i.v. with 75 K  $\gamma$ -spz followed by two weekly immunizations of 20 K  $\gamma$ -spz.  $\gamma$ -spz-immune and naive control mice were challenged i.v. 1 week later with 10 K spz.

### Determination of parasitemia

Parasitemia was determined microscopically using Giemsa on thin blood smears prepared from individual mice starting day 3 after challenge.

### Preparation of KC

IHMC were isolated from livers [7] and incubated with anti-CD8 and -CD4-conjugated magnetic beads, washed and then applied to a MS magnetic column. The flow through cells were labeled with biotinylated anti-Mac3 Ab (Cederlane, Ontario, Canada) and anti-biotin beads (Miltenyi Biotec), and applied to a second MS column. After removal of unlabeled cells, the bound Mac3<sup>+</sup> KC were eluted.

### Phagocytosis

Mac3<sup>+</sup> KC were analyzed for phagocytic activity using Vybrant<sup>TM</sup> Phagocytosis Assay Kit (V-6694) (Molecular Probes, OR) according to manufacturer's instructions.

### Flow cytometry

Cells were incubated in PBS/NMS with Fc block (BD PharMingen, San Diego, CA) for 20 min and then with the relevant mAb at 4°C for 30 min, and analyzed on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). The following mAb were used: PE-anti Mac 3(553324), FITC-anti CD40 (553790), FITC-anti CD80 (553768), FITC-anti CD86 (553691), FITC-anti H-2K<sup>b</sup> (553569), FITC-anti I-A<sup>b</sup> (553551) (BD PharMingen), and APC-anti F4/80 (RM2905) (CALTAG, Burlington, CA). Isotype-matched mAb were used as controls.

### In vitro exposure of KC to spz

KC ( $2 \times 10^5$ /well) were added to a 96-well plate and incubated at 37°C for 6 h with infectious spz ( $2 \times 10^5$ ),  $\gamma$ -spz ( $2 \times 10^5$ ) or culture medium alone. KC were washed in PBS and stained with PE-anti-Mac3 and FITC-anti-H-2K<sup>b</sup> mAb and analyzed by flow cytometry.

### Cytokines

KC ( $1 \times 10^5$ /well) were incubated at 37°C for 3 h in a 96-well plate. Nonadherent cells were removed by washing and the adherent cells were cultured overnight in 200  $\mu$ l of complete medium (CM) ((RPMI-1640, 10% FBS (Gibco BRL, Rockville, MD), Glutamax (Gibco BRL), penicillin/streptomycin (Gibco BRL) and  $5 \times 10^{-5}$  2-ME (Sigma-Aldrich))  $\pm$  LPS (200 ng/ml). The supernatants were frozen at -70°C until use. IL-10 and IL-12 were measured using BD OptEIA ELISA Kits (BD PharMingen) according to manufacturer's instructions.

### ELISPOT

Plates were coated with either anti-IL-4 (Bio Source, Camarillo, CA) or anti-IFN- $\gamma$  (Bio Source) capture Ab in PBS and then blocked with 1% milk/PBS. IHMC ( $2 \times 10^5$ /200  $\mu$ l) in CM were added/well and cultured overnight at 37°C. The plates were washed with PBS/Tween and the detection Ab plus alkaline phosphatase Strep Avidin (Bio Source) were added for 2 h at room temperature. After repeated washing, the spots were revealed with NBP/BCIP (Bio-Rad, Hercules, CA) and enumerated using a computerized image analyzer (3.2 Scanalytics, Fairfax, VA) and IP Lab software. IL-4 and INF- $\gamma$  *ex vivo* responses were scored as the mean  $\pm$  SD in triplicate wells.

### RT-PCR

RNA was purified from KC lysed in TRIzol (Invitrogen, Carlsbad, CA) and 5  $\mu$ g of total RNA was reverse transcribed using Superscript II according manufacturers instructions.

Mouse genomic DNA was purified from spleens using DNA STAT 60 (Iso - Tex Diagnostics, INC, Friendswood, TX) according to manufacturers instructions and diluted to 100 ng/ $\mu$ l.

Quantitative RT-PCR reactions were established (Quaigen, Valencia, CA) with the following primers (Invitrogen): GAPDH Forward Primer: TCCCTCACAATTTCATCCCC, GAPDH Re-

verse Primer: CCCTAGGCCCTCCTGTTATT, H2-K<sup>b</sup> Forward Primer: AGTCCACCCCTCTACACCAGG, H2-K<sup>b</sup> Reverse Primer: AGGGAAGACAGAGCAGTGCAG, using incorporation of cybergreen as the read out. Assays were done in 96-well plates; detection of PCR products was performed using an ABI 7700 (PE Applied Biosystems, Foster City, CA) at the following thermocycling conditions: 1 cycle at 95°C for 10 min and 50 cycles of 95°C for 15 s and 55°C for 1 min.

Standard curves were generated from a DNA standard and the amount of cDNA was determined for each sample. Quantitative H-2K<sup>b</sup> gene expression was done in triplicate and was expressed as a ratio of H-2K<sup>b</sup> over GAPDH for each sample.

### Antigen-presentation assays

KC were pulsed with either OVA (22 µM) (Sigma, MW 44.28 kDa) or SIINFEKL (0.1 µM) (Alpha Technologies, San Antonio, TX) in serum free medium for 60 min at 37°C. Ag-pulsed KC (1 × 10<sup>5</sup> cells/well) were co-cultured with T hybridoma cells (1 × 10<sup>5</sup>) (RF33.70, K<sup>b</sup>-restricted, OVA<sub>257–264</sub>-specific) (gift from Dr. K Rock) in a 96-well plate at 37°C for 72 h. IL-2 was quantified in supernatants (two wells per sample) using an IL-2 capture ELISA (BD OptEIA ELISA IL-2 Kit, BD PharMingen).

### Statistical analyses

Data points were compared by the Student's *t*-test and values of *p* < 0.01 were considered significant.

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