1 Development of a Novel CD4⁺ TCR Transgenic Line that Reveals a
2 Dominant Role for CD8⁺ DC and CD40-Signaling in the Generation of
3 Helper and CTL Responses to Blood Stage Malaria
4
5 Short title: Development of a Novel CD4⁺ TCR Transgenic Line to
6 Malaria

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Abstract

2

3 We describe an MHC II (IA^b)-restricted T cell receptor (TCR) transgenic mouse line
4 that produces CD4⁺ T cells specific for *Plasmodium* species. This line, termed PbT-II,
5 was derived from a CD4⁺ T cell hybridoma generated to blood-stage *Plasmodium*
6 *berghei* ANKA (PbA). PbT-II cells responded to all *Plasmodium* species and stages
7 tested so far, including rodent (PbA, *P. berghei* NK65, *P. chabaudi* AS and *P. yoelii*
8 17XNL) and human (*P. falciparum*) blood-stage parasites as well as irradiated PbA
9 sporozoites. PbT-II cells can provide help for generation of antibody to *P. chabaudi*
10 infection and can control this otherwise lethal infection in CD40L-deficient mice.
11 PbT-II cells can also provide help for development of CD8⁺ T cell-mediated
12 experimental cerebral malaria (ECM) during PbA infection. Using PbT-II CD4⁺ T
13 cells and the previously described PbT-I CD8⁺ T cells, we determined the dendritic
14 cell (DC) subsets responsible for immunity to PbA blood-stage infection. CD8⁺ DC (a
15 subset of XCR1⁺ DC) were the major antigen presenting cell (APC) responsible for
16 activation of both T cell subsets, though other DC also contributed to CD4⁺ T cell
17 responses. Depletion of CD8⁺ DC at the beginning of infection prevented ECM
18 development and impaired both Th1 and Tfh responses; in contrast, late depletion
19 did not affect ECM. This study describes a novel and versatile tool for examining
20 CD4⁺ T cell immunity during malaria and provides evidence that CD4⁺ T cell help,
21 acting via CD40L signalling, can promote immunity or pathology to blood stage
22 malaria largely through antigen presentation by CD8⁺ DC.

23

1 Introduction

2

3 Despite intervention strategies, malaria killed almost half a million people in 2015 (1).
 4 Murine models for malaria present similarities with human infections and allow for the
 5 detailed study of immunological processes of potential relevance to human disease (2-8).
 6 T cell receptor (TCR) transgenic murine lines specific for pathogen-derived antigens are
 7 powerful tools for studying the mechanisms involved in the development of immune
 8 responses during infection. Their ease of use and potential for manipulation offers a much
 9 broader range of opportunities for the study of T cell responses than are feasible using the
 10 endogenous T cell repertoire.

11

12 The lack of TCR transgenic mouse lines specific for *Plasmodium* antigens led to the
 13 generation of transgenic malaria parasites expressing model antigens, such as PbTG and
 14 OVA-PbA (2, 4, 9, 10), for which widely used murine T cell lines such as OT-I and OT-II
 15 could be used to monitor specific T cell responses. While the use of these parasites in
 16 conjunction with model T cell lines has aided the study of anti-malarial CD4⁺ and CD8⁺ T
 17 cell responses (6, 11-15), wild-type parasites and transgenic T cells capable of recognizing
 18 authentic parasite-derived antigens are preferred as they more closely resemble
 19 endogenous responses to natural infections. With this in mind, we recently generated a
 20 murine T cell receptor (TCR) transgenic line of *P. berghei* ANKA (PbA)-specific CD8⁺ T
 21 cells, termed PbT-I (8, 16). Here, we introduce a new MHC II-restricted (IA^b) TCR murine
 22 line, termed PbT-II, that responds to a parasite antigen expressed across multiple rodent
 23 and human *Plasmodium* species, making it a general tool for studying malaria immunity in

1 C57BL/6 (B6) mice. PbT-II TCR transgenic mice add to the existing I-E^d-restricted B5
2 TCR transgenic mice (2, 4, 17) to extend the set of available tools for the analysis of CD4⁺
3 T cell responses to parasites during *Plasmodium* infection of B6 mice.

4

5 CD4⁺ T cells orchestrate both humoral and cellular adaptive immune responses against
6 pathogens. Cross-talk between CD4⁺ T cells and naïve B cells resulting in Ig class
7 switching is essential for the clearance of certain pathogens such as *P. chabaudi* AS. Thus,
8 mice lacking CD4⁺ T cells or B cells are unable to control parasitaemia in this model (17).

9 Another important role for CD4⁺ T cells is the provision of help resulting in the licensing
10 of dendritic cells (DC) for the effective priming of CD8⁺ T cells. However, while CD4⁺ T
11 cell help is essential for primary responses to certain pathogens, such as herpes simplex
12 virus (HSV) (11, 18), it is dispensable during infection with influenza A virus, lymphocytic
13 choriomeningitis virus or *Listeria monocytogenes* (14, 19-21). It is understood that in the
14 latter cases, sufficient engagement of receptors for pathogen associated molecular patterns
15 (PAMP) on DC by material derived from the infectious agent (6, 22), or cytokines secreted
16 by innate cells upon recognition of the pathogen (23, 24), bypass the need for CD4⁺ T cell
17 help. In the case of PbA infection, the helper dependence of CD8⁺ T cell responses has not
18 been directly addressed. PbA infection of B6 mice leads to the development of
19 experimental cerebral malaria (ECM), a pathology mediated by CD8⁺ T cells that is used
20 as a model for human cerebral malaria (25). Therefore, dissection of the mechanisms that
21 lead to CD8⁺ T cell activation in this model is of importance to better understand this
22 pathology. ECM was abolished when CD4⁺ T cells were depleted during the early stages
23 of the infection in a number of studies (26-30), suggesting that CD8⁺ T cell priming relies

1 on CD4⁺ T cell help. Indeed, depletion of CD4⁺ T cells during infection with the transgenic
2 malaria parasite PbTG resulted in diminished OT-I cell proliferation (31). However,
3 transfer of OT-I cells into PbTG-infected RAG mice, lacking T cells, resulted in the
4 development of ECM in the absence of CD4⁺ T cells (9). The role of CD4⁺ T cells in
5 activating DC for priming CTL responses during blood stage PbA infection therefore
6 remained to be conclusively defined.

7

8 DC are effective antigen presenting cells (APC) that prime T cells for the generation of
9 pathogen-specific adaptive immune responses. The DC compartment is comprised of a
10 heterogeneous array of cell subsets with marked functional specialization (32).
11 Determining the relative contribution of DC subsets to T cell priming is of great importance,
12 especially for the development of new generations of vaccines in which antigen is delivered
13 to a specific DC subset seeking induction of optimal T cell responses. CD8⁺ DC possess
14 specialised machinery to perform antigen cross-presentation to CD8⁺ T cells, which other
15 conventional DC lack (33, 34). However, both CD8⁺ and CD8⁻ DC are capable of antigen
16 presentation to CD4⁺ T cells via MHC class II (35, 36). Consistent with these observations,
17 CD8⁺ DC from mice infected with PbTG were significantly more effective than CD8⁻ DC
18 at presenting OVA antigen to CD8⁺ T cells *in vitro*, whereas both DC subsets were capable
19 of priming CD4 T cells (9). Also, depletion of CD8⁺ DC in PbA infected mice resulted in
20 a sharp decrease in the numbers of activated endogenous CD8⁺ T cells in the spleen, and
21 CD4⁺ T cell activation was also impaired (37). A similar result was obtained when CD8⁺
22 DC were depleted in *P. chabaudi* infected mice (38). Another study analysing CD4⁺ T cell
23 responses to *P. chabaudi* antigen showed CD8⁺ DC as superior to CD8⁻ DC at MHC II

1 presentation in the steady state, but the latter were more efficient during infection (39). In
 2 the *P. chabaudi* model, mice devoid of CD8⁺ DC developed higher peaks of parasitaemia
 3 and more pronounced relapses than their wild-type (WT) counterparts (40). These results
 4 suggested an important role for CD8⁺ DC in the development of T cell responses against
 5 blood stage malaria parasites.

6

7 Here we introduce a CD4⁺ T cell transgenic mouse line specific for a malaria parasite
 8 antigen, termed PbT-II, that is cross-reactive with a broad range of malaria parasites
 9 including PbA, *P. chabaudi*, and *P. yoelii*, and even the human parasite *P. falciparum*.
 10 These cells are also cross-reactive, albeit weakly, to PbA sporozoites. Therefore, PbT-II
 11 cells are broadly applicable for the study of CD4⁺ T cell function in multiple malaria
 12 models.

13

1 **Materials and methods**

2 Ethics statement

3 All procedures were performed in strict accordance with the recommendations of
4 the Australian code of practice for the care and use of animals for scientific purposes. The
5 protocols were approved by the Biochemistry & Molecular Biology, Dental Science,
6 Medicine (RMH), Microbiology & Immunology, and Surgery (RMH) Animal Ethics
7 Committee, University of Melbourne (ethic project IDs: 0810527, 0811055, 0911527,
8 1112347, 1513505).

9

10 Mice, mosquitos and parasites

11 C57BL/6 (B6) mice, MHC I^{-/-} mice (41), IAE^{-/-} mice (MHC II-deficient) (42),
12 Batf3^{-/-} (43), IRF8^{-/-} (44), XCR1-DTRvenus (45), CD11cDTR (46), CD40^{-/-} (47) and
13 CD40L^{-/-} (48) mice and the transgenic strains OT-II (49), gDT-II (50), PbT-I (16) and PbT-
14 II were used between 6-12 weeks and were bred and maintained at the Department of
15 Microbiology and Immunology. Batf3^{-/-} mice, kindly provided by Kenneth M. Murphy
16 (Washington University), were backcrossed 10 generations to B6 for use in this study.
17 Transgenic T cell lines were crossed to mice expressing GFP ubiquitously (uGFP) or with
18 CD45.1 (Ly5.1) mice to allow for the differentiation of these cells from endogenous T cells
19 after adoptive transfer into recipient Ly5.2 B6 mice. XCR1-DTRvenus and CD11cDTR
20 mice were treated with diphtheria toxin (Calbiochem) as indicated in figure legend. The
21 University of Melbourne. Animals used for the generation of the sporozoites were 4-5 week
22 old male Swiss Webster mice purchased from Monash Animal Services (Melbourne,

1 Victoria, Australia) and maintained at the School of Botany, The University of Melbourne,
2 Australia.

3 *Anopheles stephensi* mosquitoes (strain STE2/MRA-128 from The Malaria
4 Research and Reference Reagent Resource Center) were reared and infected with PbA as
5 described (51). Sporozoites were dissected from mosquito salivary glands (52),
6 resuspended in cold PBS and irradiated with 2×10^4 rads using a gamma ^{60}Co source. $5 \times$
7 10^4 radiation-attenuated PbA sporozoites (RAS) in 0.2 ml of PBS were intravenously (i.v.)
8 administered to recipient mice.

9 The rodent malaria lines *Plasmodium berghei* ANKA clone 15cy1 (PbA), *P.*
10 *berghei* NK65, *P. chabaudi* AS and *P. yoelii* 17XNL were used in this study. Unless
11 otherwise stated, mice were infected i.v. with 10^4 PbA, *P. berghei* NK65, *P. chabaudi* or
12 *P. yoelii* 17XNL iRBC in 0.2 ml of PBS. The human malaria parasite *P. falciparum* 3D7
13 was used in *in vitro* analysis.

14

15 Generation of transgenic PbT-II

16 Transgenic PbT-II mice were generated using the V(D)J segments of the TCR α -
17 and β -genes of a CD4 $^+$ T cell hybridoma (termed D78) specific for an unidentified blood-
18 stage PbA antigen. This hybridoma was derived from T cells extracted from the spleen of
19 a B6 mouse at day 7 after infection with PbA. 3×10^6 splenocytes from a mouse previously
20 infected with PbA were co-cultured with 5×10^5 conventional DC (extracted from the
21 spleen of FMS-like tyrosine kinase 3 receptor ligand (Flt3-L) treated B6 mice) that were
22 pre-loaded for 2 hours with 2×10^6 PbA schizont lysate as previously described (53) in
23 complete RPMI at 6.5% CO $_2$, 37°C. One week later, cultured cells were re-stimulated for

1 a week with fresh DC and PbA schizont lysate. To generate PbA-specific hybridomas, *in*
 2 *vitro* cultured cells were then fused with the BWZ36.GFP fusion partner and exposed to
 3 drug selection (54). This led to isolation of the IA^b-restricted D78 hybridoma from which
 4 PbT-II T cell receptor genes were derived. V α and V β usage by D78 was defined by
 5 FACS staining with a panel of V α TCR antibodies and the mouse V β TCR screening
 6 panel (BD Biosciences).

7 The TCR α region was amplified by PCR from the cDNA of the D78 hybridoma
 8 using the forward primer GGATCCAGGAATGGACAAGATTCTG containing a *Bam*HI
 9 recognition sequence at the 5' end, designed to bind the 5' UTR region of V α 2, and the
 10 reverse primer CAGATCTCAACTGGACCACAG containing a *Bgl*II recognition
 11 sequence at the 5' end, specific for the C α region. Sequencing analysis revealed that the
 12 TCR α -chain consisted of V α 2.7, J α 12 and C α gene segments. The V α 2.7-J α 12-C α
 13 segment was cloned into the *Bam*HI site of the pES4 cDNA expression vector (55).

14 TCR V β usage was confirmed by PCR on cDNA converted from the RNA of the
 15 D78 hybridoma using the primer GAAGATGGTGGGGCTTTCAAGGATC, specific for
 16 the V β 12 gene. Sequencing analysis revealed that the TCR β -chain consisted of V β 12, D β 2
 17 and J β 2.4. The TCR β -chain VDJ segment was amplified by PCR from the genomic DNA
 18 of the D78 hybridoma using the forward primer GGATCGATCACACTTGTTCCTCGTG
 19 specific for the V β 12, incorporating a *Cla*I restriction enzyme site at the 5' end, and the
 20 reverse primer GATCGATCAGCTCACCTAACACGAGGA specific for J β 2.4 and
 21 incorporating a *Cla*I site at the 5' end and sequenced. The V β 12-D β 2-J β 2.4 segment was
 22 found to contain a *Cla*I within its sequence, so a new segment was synthesised in which
 23 the *Cla*I site (ATCGAT, coding for the aminoacids Asp and Arg) was changed for

1 ATCGAC (no change in aminoacid sequence). The new segment was cloned into the
2 unique *Cla*I restriction site of the p3A9C β TCR genomic expression vector (49).

3 Alpha- and beta-chain vector sequences were removed using combined *Cla*-I/*Not*-
4 I and *Apa*-I/*Not*-I restriction enzyme digest, respectively, and coinjected into blastocysts of
5 B6 mice to generate transgenic founder mice.

6

7 Dendritic cell isolation

8 Dendritic cells were purified from the spleens of mice as previously described (9).

9 Briefly, spleens were finely minced and digested in 1 mg/ml collagenase 3 (Worthington)
10 and 20 μ g/ml DNase I (Roche) under intermittent agitation for 20 min at room temperature.

11 DC-T cell complexes were then disrupted by adding EDTA (pH 7.2) to the digest to a final
12 concentration of 7.9 mM and continuing the incubation for 5 more min. After removing
13 undigested fragments by filtering through a 70 μ m mesh, cells were resuspended in 5 ml
14 of 1.077 g/cm³ isosmotic nycodenz medium (Nycomed Pharma AS, Oslo, Norway),
15 layered over 5 ml nycodenz medium and centrifuged at 1700 x g at 4°C for 12 min. In the
16 experiments to determine MHC restriction by the hybridomas, the light density fraction
17 was collected and DC were negatively enriched by incubation with a cocktail of rat
18 monoclonal anti-CD3 (clone KT3-1.1), anti-Thy-1 (clone T24/31.7), anti-Gr1 (clone
19 RB68C5), anti-CD45R (clone RA36B2) and anti-erythrocyte (clone TER119) antibodies
20 followed by immunomagnetic bead depletion using BioMag goat anti-rat IgG beads
21 (Qiagen).

22 In the experiments involving analyses of DC subsets, cells obtained after
23 centrifugation in nycodenz medium were stained for CD11c, MHC II, CD8 and CD4 and

sorted into (MHC II^{hi} CD11c^{hi}) CD8⁺CD4⁻ (CD8 DC), CD8⁻CD4⁺ (CD4 DC) or CD8⁻CD4⁻ (double-negative, DN DC) using a FACS Aria III sorter (BD Biosciences).

DC were resuspended in complete DMEM medium supplemented with 10% foetal calf serum (FCS) before use in functional assays.

Functional assay with hybridomas and IL-2 ELISA

For *in vitro* stimulation assays, DC (5×10^4 per well) were cultured for 1h with titrated amounts of lysed whole blood containing mixed stages of PbA parasites before adding 5×10^4 D78 or PbA-specific MHC I-restricted B4 hybridoma cells [16]. For *ex vivo* experiments, DC were extracted from the spleens of PbA infected mice on day 3 after infection with 10^6 iRBC.

After culture for 40h at 37°C in 6.5% CO₂, supernatants were collected and concentrations of IL-2 were assessed using the Mouse IL-2 ELISA Ready-Set-Go kit (eBiosciences) following manufacturer's instructions. Data were represented using logarithmic scales. Because it would not be possible to represent concentration values of "0" in logarithmic scales, all data values were increased by 1. The detection limit of the Mouse IL-2 ELISA Ready-Set-Go kit is 2 pg/ml.

The calculation of the net contribution of DC subsets to MHC II presentation was done by multiplying the mean IL-2 values induced by CD4⁺, CD8⁺ or DN DC in Fig 5B by the relative abundance of the corresponding DC subset in the spleen (in our experiments, MHC II^{high} CD11c^{high} cells in the spleen contained 55.1% CD4⁺ DC, 13.4% DN DC and 17.7% CD8⁺ DC on average). The numbers obtained from all three DC subsets, representing the IL-2 stimulation corrected to relative DC abundance, were added and the

percentage of each individual DC subset in the total number obtain was calculated. This was done for all five DC numbers tested in Fig 5B (3.125×10^3 to 50×10^3 DC per well) and the average of the five values obtained for each DC subset was calculated.

4

5 Red Blood Cell coating

Blood was collected from a naive B6 donor. After estimating the concentration of RBC, blood was washed by adding 10 ml DMEM and centrifuging at 3750 rpm for 10 min at 4°C (with mild braking). The pellet was then diluted in a solution of DMEM containing 100 mg/ml OVA (Sigma) at a concentration of 10^5 RBC/ μ l and incubated at 37°C for 30 min. Cells were washed 3 times in PBS before being added to the DC cultures.

To coat RBC with PbA antigen, the concentration of parasites in a blood sample of an infected B6 donor mouse was estimated before lysing by 3 consecutive cycles of freezing/thawing in liquid nitrogen followed by passage through a 30G needle 6 times. The equivalent of 1 parasite/RBC was added to the RBC/OVA solution.

15

16 Generation of bone marrow chimeras

B6 mice were irradiated with two doses of 550 rads 3 hours apart and reconstituted 4h later with 5×10^6 bone marrow cells. These were collected from B6 and CD11c-DTR donor mice and depleted of T cells by coating with antibodies against CD4 (RL172), CD8 (3.168) and Thy1 (J1j) followed by incubation with rabbit complement for 20 min at 37°C. One day later, mice were injected intraperitoneally (i.p.) with anti-Thy1 antibody (T24) to deplete residual T cells. Chimeric mice were rested for 8-10 weeks before use, receiving

1 water containing 2.5 g/L neomycin sulphate and 0.94 g/L polymyxin B sulphate for the
2 first 6 weeks after irradiation.

3

4 T cell isolation and *in vivo* proliferation assay

5 CD4⁺ or CD8⁺ T cells were negatively enriched from the spleens and lymph nodes
6 of PbT-I/uGFP, PbT-II/uGFP, gDT-II/Ly5.1 or OT-II/Ly5.1 transgenic mice as previously
7 described (56) and labelled with CellTraceTM Violet (CTV) or carboxyfluorescein
8 succinimidyl ester (CFSE) following manufacturers instructions (ThermoFisher). 1x10⁶
9 Purified cells were injected i.v. in 0.2 ml PBS a day before mice were infected with malaria
10 parasites (10⁴, 10⁵ or 10⁶ iRBC or 5x10⁴ RAS, as stated in the figure legend) or HSV (10⁵
11 pfu HSV). Spleens were harvested at various time points after infection for the analysis of
12 transgenic TCR cell proliferation by flow cytometry.

13 To deplete endogenous CD4⁺ T cells before adoptive transfer, mice were injected
14 i.v. with 100 µg of anti-CD4 antibody (clone GK1.5) 7 and 4 days prior to the transfer of
15 PbT-II cells.

16

17 *In vitro* proliferation assay

18 Spleens were isolated from B6 mice that had been infected with 10⁶ PbA iRBC 3
19 days earlier and DC were enriched by a 1.077 g/cm³ nycodenz density centrifugation
20 followed by negative selection. 2x10⁵ DC and 10⁵ CTV-labelled PbT-II cells were
21 incubated with titrated amounts of *P. falciparum*- or PbA-iRBC for 3 days. PbT-II
22 proliferation was assessed by flow cytometry.

23

1 Flow cytometry

2 Cells were labelled with monoclonal antibodies specific for CD8 (clone 53-6.7),
 3 CD4 (RM 4-5), Thy1.2 (30-H12), MHC II (M5/114.15.2), CD11c (N418), CD45.1 (A20),
 4 V α 2 (B20.1), V β 12 (MR11-1), V α 8.3 (B21.14), V β 10 (B21.5) or CD69 (H1.2F3), Sirp α
 5 (P84), Tbet (4B10), Bcl6 (K112-91), Foxp3 (FJK-165), GATA3 (L50-823), ROR γ t (Q31-
 6 378). Dead cells were excluded by propidium iodide staining. Cells were analyzed by flow
 7 cytometry on a FACS Canto or Fortessa (BD Biosciences), using the Flowjo software
 8 (Tree Star Inc.).

9

10 For intracellular staining, cells were permeabilised using the Transcription Factor Buffer
 11 Set (BD Biosciences) following manufacturer's instructions. Dead cells were excluded
 12 using LIVE/DEAD fixable dead cell stain (Thermofisher).

13

14 For the determination of parasitaemia, 1-2 μ l blood were collected from the tail vein,
 15 diluted in FACS buffer (containing 1% w/v BSA and 5mM EDTA) and incubated at 37°C,
 16 6.5% CO₂ with 5 μ g/ml Hoechst 33258 (Thermofischer) for 1h before running on a Fortessa.
 17 Blood from naïve mice was used as negative control (background stain usually ranged
 18 between 0.10-0.14%)

19

20 Generation and monitoring of ECM

21 Mice infected with blood-stage PbA were monitored daily for the development of
 22 ECM. Mice were considered to have ECM when showing signs of neurological symptoms
 23 such as ataxia and paralysis, evaluated as the inability of mice to self-right.

1

2 Detection of *P. chabaudi*-specific antibody

3 Nunc-Immuno™ MicroWell™ 96-well ELISA Plates (300μL round-bottom) were
4 coated overnight at 4°C with 50μL PBS containing *P. chabaudi* blood-stage lysate
5 (equivalent to 6.6×10^5 parasites/well). Unbound antigen was washed away by soaking the
6 plates in 0.05% tween 20/PBS (i.e. wash) $\times 4$ times. The wells were blocked with 50μL of
7 5% skim milk/PBS for 15-30min. Plasma samples were serially diluted in 5% skim
8 milk/PBS and incubated overnight at 4°C. The wells were washed $\times 6$ with 0.05% tween
9 20/PBS and incubated overnight at 4°C with 50μL of 5% skim milk/PBS containing anti-
10 mouse IgG-HRP (1:10,000) to assess the total IgG responses. The wells were washed $\times 6$
11 with 0.05% tween 20/PBS and HRP was detected by adding 50μL ABTS substrate in the
12 wells and incubating for 1.5-2h at RT. Optical density at 405nm (OD_{405nm}) and the
13 background at 492nm (OD_{492nm}) was determined using an ELISA plate reader. The end-
14 point titers were calculated by using cut-off values determined as $2 \times \text{SD}$ above average
15 OD_{405nm-492nm} values of control wells containing no plasma.

16 *P. chabaudi* antigen used to coat ELISA plates was prepared as follows: Blood from
17 *P. chabaudi*-infected mice was incubated with 0.05% saponin for 3 min at RT to release
18 parasites from RBC. Parasites were then broken up by snap freezing in liquid nitrogen and
19 thawing 3 times, followed by 6 passages through a 30G needle.

20

21 Statistics

22 Data were log transformed for conversion into a normal distribution and then
23 analysed using parametric statistical tests such as t-test for the comparison of 2 groups or

1 ANOVA followed by Tukey's Multiple Comparison Test for simultaneous comparison of
2 multiple groups. *P*-values <0.05 (*), <0.01 (**) or 0.001 (***) were considered statistically
3 significant.

4

1 **Results**

2

3 **Generation of an MHC II-restricted T cell hybridoma specific for PbA.**

4

5 To study the CD4⁺ T cell response to blood stage malaria, we developed an MHC II-
6 restricted T cell hybridoma specific for PbA. This hybridoma, termed D78, was derived by
7 fusing the immortalized cell line BWZ36.GFP (57) to CD4⁺ T cells isolated from the spleen
8 of a B6 mouse infected with blood stage PbA. To assess MHC restriction of D78, DC
9 deficient in MHC I or MHC II molecules were pre-incubated with lysate of PbA-infected
10 red blood cells (iRBC) and then tested for stimulatory capacity (Fig 1). Whereas both WT
11 and MHC I knockout (KO) DC were able to efficiently stimulate this hybridoma, IA^b
12 deficient (MHC II KO) DC were non-stimulatory. In contrast, MHC II KO DC were able
13 to stimulate a PbA-specific MHC I-restricted hybridoma (16), showing these DC were
14 functional (Fig S1A). Furthermore, D78 responded efficiently to PbA iRBC lysate, but
15 failed to respond to DC activated by non-specific stimuli such as LPS, polyI:C or CpG,
16 thus excluding self-reactivity or reactivity to foetal calf serum proteins (Fig S1B). Together,
17 these findings indicated that the D78 hybridoma was MHC II-restricted and specific for
18 malaria antigen.

19

20 **Generation of a PbA-specific TCR transgenic line.**

21

22 To enable monitoring of CD4⁺ T cell immunity to PbA *in vivo*, an MHC II-restricted T cell
23 receptor (TCR) transgenic mouse line was developed using the TCR alpha and beta chains

expressed by D78. This line has been termed PbT-II, consistent with our nomenclature for MHC I and II restricted OVA-specific lines OT-I and OT-II and our recently reported MHC I-restricted PbA-specific line specific, termed PbT-I (16). Analysis of the spleen and inguinal lymph node (iLN) of PbT-II mice revealed skewing towards CD4⁺ T cells as well as efficient expression of the V α 2 and V β 12 transgenes derived from D78 (Fig 2 and S1D). Total cellularity in the spleen of PbT-II mice was similar to WT mice, with CD4⁺ T cells largely compensating for reduced numbers of CD8⁺ T cells in the former (Fig S1E). Some reduction in the total cellularity of the iLN was evident, but the reason for this is unclear. Of note, increased numbers of CD4⁻ CD8⁻ (double-negative (DN)) T cells were also found in the spleen and iLN of PbT-II mice. In the thymus, PbT-II mice showed an increased number and proportion of single positive CD4⁺ T cells and double negative T cells (Fig S1C and S1E Fig). Similar to previously described TCR transgenic mice, total cellularity was reduced in the thymus, indicative of efficient positive selection (16, 58).

PbT-II cells respond to multiple *Plasmodium* species and life-cycle stages.

PbT-II mice were generated without knowledge of their specific peptide antigens. To further characterise the specificity of this line, we tested responsiveness to different species of malaria parasites. CellTraceTM Violet (CTV)-labelled PbT-II cells were adoptively transferred into B6 mice one day before infection with iRBC from either PbA, *P. berghei* NK65, *P. chabaudi* or *P. yoelii* 17XNL. Several days later, PbT-II proliferation was assessed in the spleen, revealing reactivity to all *Plasmodium* species (Fig 3A). This result prompted us to determine whether PbT-II cells also responded to the human malaria

1 parasite *P. falciparum*. *In vitro* culture of PbT-II cells with DC and blood-stage *P.*
 2 *falciparum* lysate resulted in PbT-II proliferation comparable to that seen in response to
 3 PbA (Fig 3B), indicating that the antigen recognised by PbT-II cells is conserved in
 4 multiple *Plasmodium* species.

5

6 We then asked whether the antigen recognized by PbT-II cells was also expressed by pre-
 7 erythrocytic stage PbA parasites. To examine this, CTV-labelled PbT-II cells were
 8 adoptively transferred into B6 mice that were then infected with radiation-attenuated
 9 sporozoites (RAS) that do not develop into blood stage infection. This revealed significant,
 10 though weak proliferation of PbT-II cells 6 days later, indicating some reactivity to the pre-
 11 erythrocytic stage of PbA (Fig 3C).

12

13 Because the PbT-II cells proliferated in every assay we performed, we wanted to confirm
 14 *in vivo* that such responsiveness was due to the recognition of a malaria parasite-derived
 15 epitope, and not to non-specific inflammatory signals. We transferred gDT-II cells, which
 16 are CD4⁺ T cells specific for HSV, and PbT-II cells into B6 mice that were intravenously
 17 infected with 10⁵ HSV plaque-forming units (pfu) one day later. Whereas gDT-II cells
 18 proliferated extensively by day 10 after infection, PbT-II cells remained undivided (Fig
 19 S1F).

20

21 Taken together, these results revealed a broad responsiveness of PbT-II cells to malaria
 22 parasites, including different life-cycle stages and species, showcasing the potential for
 23 these T cells as tools to broadly study CD4⁺ T cell responses in malaria.

1

2 **PbT-II cells can provide immunity to *P. chabaudi* infection.**

3

4 Both B cells and helper T cells are essential for the control of *P. chabaudi* parasitaemia
 5 (59, 60) and protection is mainly achieved by parasite-specific T cell-dependent antibodies
 6 (17). This model was therefore ideally suited to test whether PbT-II cells have the capacity
 7 to provide protective immunity to malaria. We transferred PbT-II cells into CD40L-
 8 deficient mice, in which endogenous CD4⁺ T cells are unable to provide help to B cells
 9 (47), and infected these mice with 10⁴ *P. chabaudi* iRBC. CD40L-deficient mice lacking
 10 PbT-II cells succumbed to infection during the peak of parasitaemia on day 9 p.i., or shortly
 11 thereafter, whereas those adoptively transferred with PbT-II cells survived >30 days
 12 (Figure 4A, S2). These latter mice developed a second peak of parasitaemia on day 19 p.i.
 13 that occurred earlier and was higher than that of wild-type (WT) mice (Fig 4B and S2).
 14 Although parasitaemia was still detectable on day 26 after infection, CD40L-deficient mice
 15 that received PbT-II cells had recovered from disease symptoms, as revealed by increased
 16 body weight to pre-infection levels (Fig S2), and were sacrificed >50 days after infection
 17 without signs of disease. Analysis of the plasma of these mice on day 9 after infection also
 18 revealed increased levels of *P. chabaudi*-specific IgG in CD40L-deficient mice that
 19 received PbT-II cells compared to those that did not receive cells (Fig 4C).

20

21 The protective effect of PbT-II cells for *P. chabaudi* infection was not limited to the
 22 production of antibodies, as adoptive transfer of PbT-II cells into RAG1-deficient mice,
 23 which are devoid of T cells and B cells, also resulted in a significantly prolonged survival

(Fig 4A). The first peak of parasitaemia, on day 9 after infection, was significantly higher in RAG1-deficient mice that did not receive PbT-II cells than in WT mice and RAG1-deficient mice that received PbT-II cells (Fig 4B and S2). Unlike as seen for CD40L-deficient mice, however, PbT-II cells did not enable RAG1-deficient mice to fully control parasitaemia and these mice eventually succumbed to infection (Fig 4A, B and S2).

These results clearly show PbT-II cells promoted immunity against *P. chabaudi* infection via antibody production and antibody-independent mechanisms that contributed to the control of parasitaemia.

CD8⁺ Dendritic Cells are the main antigen presenting cells for PbT-II cells.

Previous reports using DC from infected mice *ex vivo* have implicated both CD8⁺ and CD8⁻ DC in MHC II-restricted antigen presentation during blood-stage malaria infection (9, 39, 61). To examine the capacity of conventional DC (cDC) subsets to present malaria antigens to CD4⁺ T cells, we enriched cDC (defined as CD11c^{high} MHC class II^{high} cells) from the spleens of naïve mice and subdivided them into three subsets: CD8⁺CD4⁻, CD8⁻CD4⁺ and CD8⁻CD4⁻ DC (CD8⁺, CD4⁺ and DN DC respectively) (62). CD8⁺ DC also express XCR1 (45), Clec9A (63), CD24 and DEC205 (62), and lack expression of Sirpα (Fig S3A). CD4⁺ DC are the most abundant of the three subtypes (making up around 55% of all cDC), and express CD11b (62) and Sirpα (Fig S3A). DN DC are a heterogeneous group of DC in which most cells express Sirpα and CD11b, but a small group lack Sirpα (Fig S3A), likely cDC precursors(64). We incubated purified DC subtypes with PbA iRBC and the D78 hybridoma *in vitro* and assessed the hybridoma responses (Fig 5A). While all three DC

1 subsets were capable of stimulating D78, their efficacy varied considerably: CD8⁺ DC
 2 induced the strongest responses; DN DC had an intermediate stimulatory capacity and
 3 CD4⁺ DC were significantly less efficient than any of the other subtypes. A similar result
 4 was obtained when the three cDC subsets were purified from the spleens of B6 mice that
 5 had been infected with PbA 3 days earlier (Fig 5B). In this case, the stimulation exerted by
 6 DN DC and CD4⁺ DC was comparable and significantly lower than that of CD8⁺ DC.

7
 8 It was possible that the observed superiority of CD8⁺ DC was due to the characteristics of
 9 the specific peptide recognised by the D78 hybridoma, and not generalizable to other
 10 peptides. To clarify this point, we sought to test the capacity of cDC subsets at presenting
 11 OVA to the OT-II hybridoma *in vitro*. Consistent with previous studies (34), incubation of
 12 CD4⁺, CD8⁺ and DN DC with soluble OVA demonstrated a comparable capacity of all
 13 these DC to stimulate the OT-II hybridoma (Fig 5C). However, because the form of the
 14 antigen influences the effectiveness of its capture and processing by DC (34), and the
 15 malaria parasite antigen used to assess D78 responses *in vitro* in Fig 5A was cell-associated
 16 (i.e. from iRBC), we coated RBC from a naive B6 mouse with OVA in an attempt to
 17 provide this antigen to DC in a comparable, cell-associated fashion. Similar to the
 18 dominance of CD8⁺ DC in presentation of iRBC malaria antigen to D78, these DC were
 19 the most efficient stimulators of the OT-II hybridoma when OVA-coated RBC were used
 20 (Fig 5D). CD4⁺ DC were the least efficient DC subtype and DN DC exhibited an
 21 intermediate stimulatory capacity.

22

1 Together, these results indicate that CD8⁺ DC have the highest capacity for stimulation of
2 PbT-II T cells and suggest that this DC subset is particularly efficient at presenting MHC
3 II-restricted antigens associated with RBC.

4

5 To validate the role of CD8⁺ DC *in vivo*, we then took advantage of the PbT-II TCR
6 transgenic mice, which enabled us to follow the response of adoptively transferred PbT-II
7 T cells in mice lacking DC. Although DC are efficient APC capable of activating CD4⁺ T
8 cells, other cells can present antigen via MHC II and can therefore contribute to CD4⁺ T
9 cell priming (65). To assess the contribution of DC in the priming of PbT-II cells during
10 PbA infection, we first reconstituted lethally-irradiated B6 mice with bone marrow from
11 CD11c-DTR mice. In these chimeras, DC could be depleted by the administration of
12 diphtheria toxin (DT) (46). Consistent with previous reports (9, 66), DT treatment of
13 CD11cDTR->B6 chimeras during PbA infection resulted in the complete abrogation of
14 PbT-II cell proliferation (Figs 6A and E), demonstrating an essential role of DC in CD4⁺
15 T cell priming during blood stage infection. A similar result was obtained when DC were
16 removed after *P. chabaudi* infection (Fig S3B).

17

18 To assess the role of individual DC subsets in the PbT-II response, we examined reactivity
19 in mice deficient in subsets of DC due to the lack of specific transcription factors, namely
20 Batf3 (43) or IRF8 (44). Batf3 deficient mice contain all DC subsets except CD8⁺ DC and
21 their migratory equivalents, the CD103⁺ DC, and have been shown to sustain low CD8⁺ T
22 cell proliferation after PbA infection (67),(43). However, on a B6 background, some CD8⁺
23 DC can be found in the LN of Batf3-deficient mice (68) and precursors capable of MHC

II-restricted presentation but not cross-presentation are found in the spleen (69). To examine the PbT-II response in Batf3-deficient mice, CTV-labelled PbT-II cells were adoptively transferred one day before infection with 10^4 PbA iRBC. PbT-II proliferation was then assessed 5 days later (Figs 6B and E). This showed that the total number of proliferating PbT-II cells was reduced in Batf3 deficient mice (Fig 6B), and pair-wise comparisons showed a 50% reduction relative to WT controls (Fig 6E). These findings indicated that PbT-II cells were heavily dependent upon mature $CD8^+$ DC for antigen presentation, but suggested some participation by other DC, possibly the less mature form of $CD8^+$ DC present in Batf3-deficient spleens (incapable of cross-presentation) or an alternative $CD8^-$ DC subset.

IRF8-deficient mice are profoundly deficient in $CD8^+$ DC, $CD103^+$ DC and pDC, but contain other conventional DC subsets, so these mice represent an ideal host for assessing the role of $CD8^-$ conventional DC in antigen presentation. CTV-labelled PbT-II cells were therefore adoptively transferred into IRF8-deficient mice 1 day before infection with PbA iRBC and T cell proliferation assessed 5 days later (Figs 6C and E). PbT-II proliferation was severely reduced in these mice (Fig 6C), amounting to about 20% of WT responses (Fig 6E), further supporting the major role for $CD8^+$ DC in PbT-II responses.

Lack of IRF8 is known to result in immune defects in addition to the absence of $CD8^+$ DC, e.g. $CD8^-$ DC, although in normal numbers, are hypo-responsive to microbial stimulation (70). Also, IRF8 deficient mice show an excessive production of granulocytes resulting in splenomegaly (44), which could have hindered proper priming and proliferation of PbT-II

1 cells. Thus, to further explore the importance of CD8⁺ DC in PbT-II priming to PbA, we
 2 assessed PbT-II proliferation in DT-treated XCR1-DTRvenus mice. XCR1 is a chemokine
 3 receptor expressed by mature CD8⁺ DC and by their migratory CD103⁺ counterparts, found
 4 in lymph nodes. DT treatment of XCR1-DTRvenus mice, which express the DT receptor
 5 under the XCR1 promoter, results in the elimination of CD8⁺ DC from the spleen (45).
 6 Consistent with the results obtained using IRF8-deficient mice infected with blood stage
 7 PbA, PbT-II proliferation was significantly reduced in the spleen of DT-treated XCR1-
 8 DTRvenus mice, amounting to about 30% of WT controls (Figs 6D and E).

9

10 Together, these data demonstrated an essential role for DC in the generation of PbT-II cell
 11 responses to blood stage malaria parasites and argued that the CD8⁺ DC subtype played a
 12 dominant role.

13

14 **CD8⁺ DC influence Th1 and Tfh induction**

15

16 DC subsets can influence the expression of transcription factors by T cells and drive their
 17 differentiation into particular T helper phenotypes (35, 36, 71-73). To address the role of
 18 CD8⁺ DC on the differentiation of PbT-II cells after PbA infection, XCR1-DTRvenus mice
 19 were adoptively transferred with PbT-II cells and then infected with 10⁴ PbA iRBC. These
 20 mice were either treated with DT throughout the infection to remove CD8⁺ DC or given
 21 PBS, maintaining this DC subset. On day 7, the expression of transcription factors that
 22 define Th1, Th2, Th17, Tfh and Treg subsets (i.e. Tbet, GATA3, RORγt, Bcl6 and Foxp3
 23 respectively) was assessed in PbT-II cells revealing induction of Tbet⁺ and Bcl6⁺ PbT-II

1 cells, which was impaired upon depletion of CD8⁺ DC (Fig 6F and S3C). No expression
2 of GATA3, ROR γ t or Foxp3 was detected in PbT-II cells, even in the presence of CD8⁺
3 DC. A similar dependence on CD8⁺ DC for Th1 and Tfh cell development was observed
4 for endogenous CD4⁺ T cells (Fig 6F). Of note, and agreeing with a previous study (73),
5 the proportion of Tbet⁺ CD8⁺ T cells was also markedly reduced in mice depleted of CD8⁺
6 DC. These results suggested a major role for CD8⁺ DC in promoting Tbet expression in
7 CD4 and CD8 T cells, and a lower, but significant contribution to the generation of Tfh
8 cells.

9

10 **CD8⁺ DC function early after infection is essential for ECM development**

11

12 *In vitro* experiments involving the use of transgenic parasites expressing model antigens
13 previously showed that CD8⁺ T cell priming during blood stage malaria parasite infection
14 is efficiently performed by CD8⁺ DC, but only poorly by other DC subsets (9). To confirm
15 this point *in vivo*, we transferred CD8⁺ T cells from the PbT-I transgenic line into Batf3-
16 deficient mice or XCR1-DTRvenus mice and then infected these mice with PbA. XCR1-
17 DTRvenus mice were treated with DT from days -1 to 2 after infection to specifically
18 remove CD8⁺ DC. PbT-I proliferation was severely decreased in the absence of CD8⁺ DC
19 in both cases (Fig 7A and B), confirming the important role of this DC subtype in CD8⁺ T
20 cell activation during blood stage PbA infection.

21

22 To assess the role of CD8⁺ DC in ECM, we first assessed ECM in Batf3-deficient mice
23 after infection with 10⁴ PbA iRBC (Fig 7C). In agreement with previous reports (67),

1 Batf3-deficient mice did not develop ECM, demonstrating an essential role for CD8⁺ DC
 2 in promoting pathology. To obtain a precise indication of the temporal requirement for
 3 CD8⁺ DC, we treated XCR1-DTRvenus mice with DT during the early stages of PbA
 4 infection (days -1 to 2pi) and monitored for ECM. While non-depleted mice rapidly
 5 succumbed to ECM, DT-treated mice were largely protected (Fig 7D), despite harbouring
 6 similar levels of parasitaemia (Fig S4A). To determine whether CD8⁺ DC function may
 7 be required during the effector phase of ECM, XCR1-DTRvenus mice were infected with
 8 PbA and treated with DT from day 5 post-infection (Fig 7E). In contrast to mice depleted
 9 of DC from the beginning of infection, all mice depleted just prior to ECM onset showed
 10 rapid disease onset supporting the view that CD8⁺ DC were essential for the priming but
 11 not the effector phase of ECM.

12

13 CD4⁺ T cell help acting on CD8⁺ DC is required for CD8⁺ T cell priming during blood 14 stage PbA infection

15

16 Primary CD8⁺ T cell responses can be CD4⁺ T cell dependent (18) or independent (19-21).
 17 To study the relevance of CD4⁺ T cell help during blood stage malaria, we looked at the
 18 expansion of CD8⁺ PbT-I cells in the absence of CD4⁺ T cells. We transferred 1 million
 19 CTV-labelled PbT-I cells into MHC II-deficient mice, devoid of CD4⁺ T cells, and infected
 20 them with 10⁴ PbA iRBC. PbT-I proliferation 5 days later was significantly reduced in
 21 MHC II-deficient mice compared with their WT counterparts (Fig 8A). Because elevated
 22 numbers of CD8⁺ T cell precursors can reduce helper requirements (74), transfer of a high
 23 number of PbT-I cells (1 million per mouse) in the previous experiment might have resulted

1 in an artificial enhancement of T cell proliferation in the mice lacking CD4 T cells. To
 2 clarify this point, we transferred lower numbers of PbT-I cells (10^5 , 10^4) into MHC II-
 3 deficient mice, infected them with 10^4 PbA iRBC and examined proliferation on day 5.
 4 This revealed that for this infection model, the response of PbT-I cells was similarly
 5 reduced in MHC II-deficient mice compared with WT counterparts regardless of the initial
 6 number of PbT-I cells transferred (Fig 8B).

7

8 Since signals derived from parasite material can promote DC licensing (22), we reasoned
 9 that infection with higher numbers of parasites might result in a decreased need for CD4⁺
 10 T cell help. To examine this issue, mice depleted of CD4⁺ T cells with the monoclonal
 11 antibody GK1.5 (as an alternative to MHC II-deficient mice), were adoptively transferred
 12 with 10^6 CTV-labelled PbT-I cells and then infected with 10^4 , 10^5 or 10^6 PbA iRBC. While
 13 numbers of divided PbT-I cells were significantly reduced in CD4⁺ T cell depleted mice
 14 compared with non-depleted controls for all parasite doses, the proportional difference was
 15 largest for the lower doses (Fig 8C). This indicated that the requirement for help during
 16 blood stage infection with PbA was somewhat affected by the infection dose.

17

18 Licensing of DC for CTL immunity requires the interaction of co-stimulatory molecules
 19 between activated CD4⁺ T cells and DC. CD40L (on the CD4⁺ T cell) and CD40 (on the
 20 DC) have been shown to mediate DC licensing in several infectious and non-infectious
 21 settings (75-77). To determine whether this pathway was relevant for the provision of help
 22 by CD4⁺ T cells during blood stage PbA infection, we transferred PbT-I cells into either
 23 mice lacking CD40 or CD40L and infected them with 10^4 iRBC. PbT-I proliferation and

1 total numbers were significantly reduced in the spleens of both CD40-deficient and
2 CD40L-deficient mice relative to WT controls (Fig 8D). Taken together, the results
3 demonstrated a need for CD4⁺ T cells and CD40-CD40L interactions for the development
4 of optimal CD8⁺ T cell responses during infection with blood stage malaria.

5

6 To demonstrate that CD4⁺ T cells provide help via CD40-CD40L interactions to promote
7 optimal CD8⁺ T cell activation during blood stage PbA infection, we adoptively transferred
8 10⁶ WT PbT-II cells together with 10⁶ PbT-I cells into CD40L-deficient mice (in which
9 endogenous CD4⁺ T cells were unable to license DC) and then infected these mice with
10 10⁴ iRBC. While PbT-I proliferation was reduced in CD40L-deficient mice, their response
11 was recovered to WT levels in mice that received PbT-II cells (Fig 8E). To assess the
12 capacity of PbT-II cells to help CD8⁺ T cells cause ECM, we examined the influence of
13 PbT-II cells on the onset of ECM in CD40L-deficient mice. While CD40L-deficient mice
14 showed a significant delay in ECM onset compared to WT mice, this delay was alleviated
15 by transfer of PbT-II cells (Fig 8F). Changes in the time frame for ECM development were
16 independent of parasitaemia, which was not significantly altered by the adoptive transfer
17 of PbT-II cells (Fig S4B). Taken together, these results showed that PbT-II cells were able
18 to help CD8⁺ T cells proliferate and cause ECM in CD40L-deficient mice.

19

20 In summary, our results indicate that CD8⁺ DC are the main APC priming both CD4⁺ and
21 CD8⁺ T cells during infection with blood stage PbA parasites. Using our newly generated
22 PbT-II transgenic cell line, we demonstrated that optimal expansion of CD8⁺ T cells
23 required CD4⁺ T cell help, which was provided to the DC via CD40-CD40L interactions.

1 Discussion

2 While TCR transgenic mouse lines are very useful for deciphering the mechanisms by
 3 which T cells operate and carry out their functions in different contexts of infection, the
 4 available range of these lines specific for blood stage malaria antigens is limited. Here we
 5 introduce a new CD4⁺ T cell transgenic mouse line, termed PbT-II, specific for a broad
 6 range of *Plasmodium* species and responsive to both blood stage and pre-erythrocytic stage
 7 infections, offering wide applicability for the study of CD4⁺ T cell function in multiple
 8 malaria models. To date, the only available CD4⁺ T cell receptor transgenic murine line
 9 specific for malaria parasites (*P. chabaudi*) was B5, which is restricted for I-E^d and
 10 therefore only usable in the BALB/c background. The PbT-II mice provide the opportunity
 11 to analyse *Plasmodium*-specific CD4⁺ T cells on the B6 background, for which availability
 12 of reagents and gene deficient mice is much broader. We show here that PbT-II cells are
 13 an excellent tool for studying CD4⁺ T cell function in the context of malaria immunity or
 14 pathology. When adoptively transferred into CD40L-deficient mice, in which endogenous
 15 CD4⁺ T cells are unable to fully activate B cells or DC (48, 75, 77), PbT-II cells were able
 16 to replace endogenous CD4⁺ T cells for the generation of both antibody responses, which
 17 allowed control of *P. chabaudi* parasitaemia, and CD8⁺ T cell responses, which caused
 18 ECM after PbA infection. This indicates that PbT-II cells are an invaluable tool for the
 19 study of CD4⁺ T cell biology during infection with *Plasmodium* parasites.

20

21 The specific antigen recognised by PbT-II cells is yet to be defined. The broad cross-
 22 reactivity to different *Plasmodium* species suggests that the target epitope belongs to a
 23 conserved protein, likely to have an essential function in blood stage parasites. The PbT-II

1 target protein is also expressed during the pre-erythrocytic stage, as PbT-II cells also
 2 responded to RAS. It is possible that this protein is only expressed late during the liver
 3 stage, shortly before merozoites are released to the blood (78), allowing for a small window
 4 of antigen presentation during the liver stage of the parasite. However, the limited
 5 development of RAS within hepatocytes argues against this possibility. Alternatively, the
 6 abundance of this protein during the liver stage of the parasite cycle may be lower than
 7 during the blood stage. Further studies will be required to determine the exact antigen
 8 recognized by PbT-II cells.

9

10 DC are extremely efficient at initiating T cell responses in numerous infection models. We
 11 have been able to demonstrate a pivotal role for the CD8⁺ DC subset in the priming of both
 12 CD4⁺ and CD8⁺ T cells during blood stage PbA infection. Our results on CD8⁺ T cell
 13 activation confirm previous *ex vivo* data showing a superior efficiency of CD8⁺ DC at
 14 priming OT-I cells after exposure to transgenic PbA parasites expressing SIINFEKL (9).
 15 They are also consistent with *in vivo* studies showing a reduced number of activated
 16 endogenous CD8⁺ T cells after PbA infection of mice deficient in CD8⁺ DC (37, 38, 67).
 17 As CD8⁺ DC possess specialised machinery for cross-presentation (34), this likely
 18 underlies their unique capacity to prime CD8⁺ T cells during infection by *Plasmodium*
 19 species.

20

21 CD8⁺ DC were also the main subset that stimulated D78 and PbT-II cells. To estimate the
 22 net contribution of the different DC subsets to MHC II presentation, the results obtained
 23 for D78 stimulation *ex vivo* (Fig 5B), which show that CD8⁺ DC are about 10-fold better

than CD4⁺ DC or DN DC at presentation, were adjusted to reflect the distinct abundance of sorted DC subsets in the spleen (CD4⁺ DC, 55.1%; CD8⁺ DC, 17.7%; DN DC, 13.4% of splenic CD11c^{hi} MHC II^{hi} cells). From this adjustment, we estimated that the contribution of CD8⁺ DC to antigen presentation via MHC II was 67% of the total, whereas that of CD4⁺ DC contributed 20% and DN DC contributed the remaining 13% (with the combined CD8⁻ DC subsets therefore contributing 33% of the total antigen presentation in the spleen). *In vivo* experiments using different mouse lines deficient in CD8⁺ DC to assess the contribution of CD8⁺ DC to PbT-II priming during blood stage PbA infection supported our observations with the D78 hybridoma: the number of dividing PbT-II cells in the spleen of Batf3-deficient or IRF8-deficient or DT-treated XCR1-DTRvenus mice was reduced to 45.1%, 16.6% and 31.0%, respectively. Because of the limitations of Batf3-deficient and IRF8-deficient mice in this system, with Batf3-deficient mice still containing immature DC capable of MHC II presentation (68, 69), and IRF8-deficient mice presenting additional immune defects (44, 70), the outcome using XCR1-DTRvenus mice likely best represents the net contribution of CD8⁺ DC to MHC II presentation. This value (69%) very closely aligns with the 67% contribution to MHC II presentation estimated using the D78 hybridoma.

These results clearly define CD8⁺ DC as the major APC contributing to CD4⁺ T cell priming during PbA infection. Previous work in our lab showed roughly equivalent CD4⁺ T cell priming *ex vivo* by CD8⁻ and CD8⁺ DC from BALB/c mice infected with a transgenic PbA parasite expressing various model T cell epitopes (9). In that case, responses in B6 mice could not be assessed because they were too weak (9). Consistent with our current

1 study, other reports have found an important role for CD8⁺ DC in CD4⁺ T cell activation:
 2 Clec9A-DTR mice depleted of CD8⁺ DC by injection of DT showed a markedly decreased
 3 activation of endogenous CD4⁺ T cells during PbA infection (37). Also, CD8⁺ DC were
 4 more efficient than CD8⁻ DC at presenting *P. chabaudi*-derived antigens to CD4⁺ T cell
 5 hybridomas (39). In the latter study, the superiority of CD8⁺ DC was only observed for DC
 6 derived from uninfected mice, and CD8⁻ DC extracted on day 7 after infection were more
 7 efficient at MHC II presentation than CD8⁺ DC. At that late time point, CD8⁺ DC showed
 8 greater cell death than CD8⁻ DC, which likely explained their decreased stimulatory
 9 capacity (39).

10

11 We also provided evidence that CD8⁺ DC contribute to the quantity of the T cell response,
 12 as CD8⁺ DC depletion led to the generation of reduced frequencies of Th1 and Tfh cells.
 13 This agrees with existing literature showing that the CD8⁺ DC subset preferentially
 14 generates Th1 responses over Th2 responses (35, 36) and is efficient at promoting Tfh
 15 immunity (71, 72). The consequences of this functional capacity for CD8⁺ DC, however,
 16 are likely to differ depending on the model studied. During PbA infection, for example,
 17 IFN- γ producing CD4⁺ T cells have been implicated in ECM by attracting CD8⁺ T cells to
 18 the brain (79). Thus, loss of Th1 cell development after CD8⁺ DC depletion may impact
 19 CD8⁺ T cell recruitment to this organ; however, in this example CD8⁺ DC would also be
 20 crucial for initiation of CD8⁺ T cell responses. Alternatively, control of *P. chabaudi*
 21 infection requires Th1 cells during the first peak of parasitemia (80) and Tfh cells for
 22 complete elimination of the parasite (81). Not surprisingly, the absence of CD8⁺ DC in
 23 this infection results in impaired parasite control and more pronounced relapses (40).

1

2 The reason for the observed dominance of CD8⁺ DC in MHC II presentation remains
3 unclear. The form of antigen encountered by DC appears to play a role, as in the present
4 work CD8⁺ DC only out-performed other DC subsets when antigen was provided in a RBC-
5 associated form, but not when in soluble form (Figs 5C and D). This is consistent with their
6 specialization in the capture of cell-associated antigen (82). Further studies are required to
7 elucidate the mechanisms underlying the superiority of CD8⁺ DC in MHC II presentation
8 during PbA infection.

9

10 Given the critical role of CD8⁺ T cells in the development of ECM (83), determining the
11 requirements for CD8⁺ T cell activation during blood stage malaria is of great importance.
12 Conventional DC are essential for the induction of ECM (66) and lack of ECM in XCR1-
13 DTRvenus mice treated with DT early in infection confirms the findings of others (37) that
14 implicate CD8⁺ DC in this process. This is not surprising given their central role in priming
15 both CD4⁺ and CD8⁺ T cells. Less clear is the extent at which CD4⁺ T cell licensing of
16 CD8⁺ DC is required for ECM development. Several studies have shown that anti-CD4⁺
17 antibody given around the time of PbA infection prevents ECM (26-30), consistent with
18 the view that CD4⁺ T cell help is required for the induction of CTL responses. However,
19 CD4⁺ T cells have also been implicated in CD8⁺ T cell recruitment to the brain, thus raising
20 the possibility that their depletion merely affects this facet of disease. Only one study
21 provides direct evidence for a contribution of CD4⁺ T cell help to CD8⁺ T cell proliferation,
22 where CD4⁺ T cell depletion resulted in low proliferation of OVA-specific OT-I cells after
23 infection with transgenic PbA parasites expressing the OVA epitope (31). By using our

1 PbT-I transgenic CD8⁺ T cells, we clearly demonstrated that CD4⁺ T cell help was essential
 2 for optimal CD8⁺ T cell expansion, as mice depleted of CD4⁺ T cells showed suboptimal
 3 PbT-I proliferation. These responses were dependent on CD8⁺ DC, as they were impaired
 4 when this subset of DC were depleted. Furthermore, they depended heavily upon CD40L,
 5 as mice deficient in this molecule had curtailed CD8⁺ T cell proliferation. The capacity of
 6 transferred PbT-II cells to help PbT-I cells to proliferate in CD40L-deficient mice further
 7 implicated CD40L as an important component of help that could be provided by PbT-II T
 8 cells. Interestingly, CD40 signalling was not absolutely essential for ECM development,
 9 as CD40L-deficient mice eventually developed ECM in the absence of added PbT-II T
 10 cells, though with a delayed kinetics. This contrasts a previous report that found CD40L-
 11 deficient mice were resistant to ECM (84), an observation that may be explained by
 12 differences in animal housing facilities, potentially microbiota, known to affect ECM (85).

13

14 Our work extends earlier findings by determining that CD4⁺ T cell help is required for
 15 optimal CD8⁺ T cell expansion after infection with different doses of parasites, and is
 16 relatively independent of the initial CD8⁺ T cell precursor frequency. By defining CD40
 17 signalling as an important event in DC licensing in this model, we have also identified a
 18 key checkpoint that could be targeted to limit pathogenic CD8⁺ T cell immunity to blood
 19 stage disease.

20

21 We have established that CD8⁺ DC are the main APC for CD4⁺ and CD8⁺ T cell priming,
 22 and that CD8⁺ T cell priming required CD4⁺ T cell help. It is likely that CD8⁺ DC are
 23 licensed by those CD4⁺ T cells that they prime, though CD4⁺ T cells primed on CD8⁻ DC

1 may also contribute to this process. The essential role for CD8⁺ DC in CD8⁺ T cell priming
2 and their dominant role in CD4⁺ T cell priming puts this subset at the centre of response
3 initiation to blood stage malaria parasites and suggests that strategies aimed at generating
4 T cell responses against blood stage malaria parasites will benefit from exploiting this DC
5 subtype. The findings that individuals with severe disease in malaria endemic areas present
6 with increased numbers of circulating BDCA3⁺ DC (86) (the human equivalent to mouse
7 CD8⁺ DC) and activated CD8⁺ T cells (38) relative to those with mild malaria, suggest that
8 CD8⁺ T cell activation during blood stage malaria may be driven by a similar process in
9 humans as in the mouse model. More precise knowledge of the mechanisms that drive
10 CD8⁺ DC function during malarial infections may, thus, allow us to develop more effective
11 strategies to combat this disease.

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- 6

1 **Figure legends**

2

3 **Fig 1. MHC II restriction of the D78 hybridoma.** Dendritic cells were enriched from the
4 spleens of WT B6 (filled circle), MHC I deficient (inverted triangle) or MHC II deficient
5 (upright triangle) mice and cultured for 1h with titrated amounts of lysed blood-stage PbA
6 iRBC before adding 5×10^4 D78 hybridoma cells. 40h later, IL-2 concentrations in the
7 supernatants were measured by ELISA. Data points denote mean of IL-2 concentration and
8 error bars represent SEM. Data were pooled from 2 independent experiments. Statistical
9 analysis was performed on log-transformed data using unpaired t tests. Asterisks denote
10 significant differences between WT and MHC II deficient DC (**, $P < 0.01$). No statistical
11 differences were found between WT and MHC I deficient DC.

12

13 **Fig 2. Characterization of T cells from the spleen and lymph nodes of PbT-II mice.**
14 Cells were harvested from the spleen and the lymph nodes of PbT-II transgenic or control
15 B6 (WT) mice. FACS analysis was performed to characterize the expression of CD4, CD8
16 and the transgenic TCR alpha ($V\alpha 2$) and beta ($V\beta 12$) chains. (A) Representative plots
17 showing the proportions of $CD4^+$ versus $CD8^+$ $Thy1.2^+$ cells in the spleen and inguinal
18 lymph node of PbT-II and WT mice. (B) Representative histograms showing the expression
19 of the transgenic TCR $V\alpha 2$ and $V\beta 12$ chains on the CD4 or CD8 single positive cells
20 from the spleen. Data are representative for 2 independent experiments.

21

22 **Fig 3. PbT-II cells are cross-reactive with multiple *Plasmodium* species and with liver**
23 **stage PbA.** (A) Cross-reactivity with murine malaria parasites. 10^6 CTV-labelled PbT-II

1 cells were transferred into B6 recipients one day before infection with 10^4 iRBC from
2 *Plasmodium berghei* ANKA (PbA), *P. berghei* NK65, *P. chabaudi* AS, *P. yoelii* 17XNL
3 or nothing. 5 days after *P. berghei* ANKA and *P. berghei* NK65 infection, or 7 days after
4 *P. chabaudi* AS and *P. yoelii* 17XNL infection, mice were killed and PbT-II proliferation
5 was assessed in the spleen. *Left*. Number of proliferating cells. *Right*. Representative
6 histograms showing PbT-II proliferation. Data are representative for 2 independent
7 experiments. Data were log transformed and analysed using One Way ANOVA and
8 Dunnet's Multiple comparisons test. Asterisks denote statistical differences with the
9 uninfected group (***, $P < 0.001$) (B) Cross-reactivity with *P. falciparum*. 2×10^5 Splenic
10 DC were cultured with 10^5 CFSE-coated PbT-II cells and titrated amounts of *P. falciparum*
11 (Pf)- or PbA-iRBC for 3 days. PbT-II proliferation was assessed by flow cytometry. *Left*.
12 Percentages of proliferating PbT-II cells to Pf or PbA antigen. *Right*. Representative
13 histogram showing PbT-II proliferation to Pf-iRBC (solid black line), PbA iRBC (dotted
14 line) and a negative control containing no antigen (tinted grey line). (C) Cross-reactivity
15 with liver stage PbA parasites. 10^6 CTV-labelled PbT-II cells were transferred into
16 recipient B6 mice one day before infection with 5×10^4 *P. berghei* ANKA radiation
17 attenuated sporozoites (RAS) or 10^4 PbA iRBC. 6 days later mice were killed and PbT-II
18 proliferation was assessed in the spleen. *Left*. Number of proliferating cells. *Right*.
19 Representative histograms showing PbT-II proliferation. The tinted line represents
20 uninfected control. The solid and dotted black lines show PbT-II cell proliferation in mice
21 infected with PbA RAS or iRBC respectively. Data were pooled from 2 independent
22 experiments. Data were log transformed and analysed using One Way ANOVA and
23 Tukey's Multiple comparisons test (**, $P < 0.01$; ***, $P < 0.001$)

Fig 4. PbT-II cells can elicit immunity to *P. chabaudi* infection. CD40L-deficient mice (CD40L KO), RAG-deficient mice (RAG KO) and WT control mice received either 5×10^5 PbT-II cells or no cells one day before infection with 10^4 *P. chabaudi* iRBC. (A) Survival of WT mice (n=10), CD40L-deficient mice with or without PbT-II cells (n=7 and 6 respectively) and RAG-deficient mice with or without PbT-II cells (n=8 and 7 respectively). Data were analysed using logrank test. Asterisks denote statistical difference between CD40L KO vs CD40L KO+PbT-II cells and RAG KO vs RAG KO+PbT-II cells respectively (***, $P < 0.001$). (B) Course of parasitaemia after *P. chabaudi* infection. Blood samples were examined by FACS. The dotted red line represents background staining in uninfected mice. Data were log transformed and analysed using one way ANOVA and Tukey's Multiple Comparison test. A statistical difference was found on day 9 between RAG KO and RAG KO+PbT-II (*, $P < 0.05$). No difference was found between CD40L KO and CD40L KO+PbT-II mice on day 9. Statistically significant differences between both CD40L KO+PbT-II or Rag KO+PbT-II mice and WT mice was found on day 19 (***, $P < 0.001$). (C) Parasite-specific IgG antibodies in CD40L KO mice. *P. chabaudi*-specific IgG end-point titers were determined on day 9 after infection using ELISA. Cut-off values were determined as $2 \times \text{SD}$ above average $\text{OD}_{405\text{nm}-492\text{nm}}$ values of control wells containing no plasma. Data were log transformed and analysed using a One Way ANOVA and Tuckey's Multiple Comparison Test (***, $P < 0.001$). The dotted line represents the limit of detection of the assay.

21

Fig 5. CD8^+ DC are more efficient than other DC subtypes at stimulating D78 hybridoma cells *in vitro* and *ex vivo*. (A) D78 stimulation by DC subtypes *in vitro*. 5×10^4

sorted CD4⁺, CD8⁺ or DN DC from naive B6 mice were pre-incubated for 1h with titrated amounts of PbA iRBC. 5x10⁴ D78 hybridoma cells were then added to the culture and incubated for a further 40h before supernatants were collected and IL-2 concentrations measured by ELISA. (B) D78 stimulation by DC subtypes *ex vivo*. 5x10⁴ sorted CD4⁺, CD8⁺ or DN DC from the spleens of B6 mice infected with 10⁶ PbA iRBC 3 days earlier were incubated with 5x10⁴ D78 hybridoma cells for 40h before supernatants were collected and IL-2 concentrations measured by ELISA. (C) Presentation of soluble OVA antigen to the OT-II hybridoma. 5x10⁴ sorted CD4⁺, CD8⁺ or DN DC from naive B6 mice were pre-incubated for 1h with titrated amounts of soluble OVA. 5x10⁴ OT-II hybridoma cells were then added to the culture and incubated for a further 40h before supernatants were collected and IL-2 concentrations measured by ELISA. (D) CD8⁺ DC are more efficient than other DC subtypes at presenting RBC-associated antigen to the OT-II hybridoma *in vitro*. 5x10⁴ sorted CD4⁺, CD8⁺ or DN DC from naive B6 mice were pre-incubated for 1h with OVA-coated RBC from a naive B6 mouse. 5x10⁴ OT-II hybridoma cells were then added to the culture and incubated for a further 40h before supernatants were collected for assessment of IL-2 concentrations by ELISA. Data were pooled from 3 independent experiments in A-C and from 4 independent experiments in D. Data were log-transformed and statistically analysed using One-way ANOVA (*, P<0.05; **, P<0.01; ***, P<0.001).

19

Fig 6. PbT-II priming during blood stage PbA infection is exclusively done by DC, among which the CD8⁺ subset is the major contributor. 10⁶ CTV-labelled PbT-II cells were adoptively transferred into different DC deficient recipient mice or WT controls, which were infected with 10⁴ PbA iRBC 1-2 days later. Proliferation of PbT-II cells was

1 measured in the spleen on day 5 after PbA infection. Total divided cells in the spleen (upper
2 graph) and the corresponding representative histograms for proliferation (below) are shown
3 in each subfigure. The different groups in the upper graph are represented in the histograms
4 below as solid black lines (DC deficient mice), dotted lines (WT control mice) and tinted
5 grey lines (uninfected mice). (A) PbT-II proliferation in DT-treated B6->B6 or CD11c-
6 DTR->B6 chimeras. Mice were treated with 100 µg DT i.p. on days -1, 1 and 3 after
7 infection. (B) PbT-II proliferation in WT or Batf3 KO mice. (C) PbT-II proliferation in
8 WT or IRF8 KO mice. (D) PbT-II proliferation in DT-treated WT or XCR1-DTRvenus
9 mice. Mice were treated with 500 µg DT i.p. on days -1, 0, 1 and 2 after infection. Data
10 were pooled from 2 independent experiments in A and D, and 3 independent experiments
11 in B and C. For statistical analysis, data were log transformed and analysed using one way
12 ANOVA followed by Tukey's Multiple comparisons test (n.s.=not significant; *, P<0.05,
13 **, P<0.01, ***, P<0.001). (E) Percentages of PbT-II proliferation in DC deficient mice
14 compared with their WT counterparts. Data derived from A-D was normalized to facilitate
15 comparisons between individual experiments. First, the average of divided PbT-II cells in
16 WT mice in each experiment was calculated. That number was then used to calculate the
17 percentage of PbT-II divided cells in both KO mice (CD11cDTR->B6, light grey circles;
18 Batf3 KO, black circles; IRF8 KO, dark grey circles; XCR1-DTRvenus mice, white circles)
19 or their respective WT counterparts (whose normalised proliferation would therefore
20 average 100%). Data in E were statistically compared using Kruskal-Wallis tests followed
21 by Dunn's multiple comparisons test for differences between the individual groups (***,
22 P<0.001). (F) Transcription factor expression in splenic PbT-II cells and endogenous CD4⁺
23 and CD8⁺ T cells primed in the presence or absence of CD8⁺ DC. XCR1-DTRvenus mice

1 received 5×10^5 PbT-II/uGFP cells and were infected with 10^4 PbA iRBC one day later.
 2 These mice were treated with either DT (500 μ g/dose) or PBS on days -1, 0, 1, 2, 4 and 6
 3 p.i. and killed on day 7. Data points represent individual mice that were pooled from 2
 4 independent experiments, log transformed and statistically analysed using an unpaired t
 5 test (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$). Data are represented as mean $\pm$ SEM.

6

7 **Fig 7. CD8⁺ DC are critical for CD8⁺ T cell priming and ECM development after PbA**

8 **infection.** (A) PbT-I proliferation in Batf3-deficient mice. WT B6 or Batf3-deficient (Batf3
 9 KO) mice were infected with 10^4 PbA iRBC and 2 days later 10^6 CFSE-labelled PbT-I
 10 cells were adoptively transferred. 3 days later, spleens were harvested and analysed for
 11 proliferation of PbT-I cells. *Left.* The proliferation of PbT-I cells pooled from 3
 12 independent experiments with 2-3 mice in each experiment. Data were log transformed and
 13 PbT-I proliferation in infected WT vs Batf3-deficient groups was compared using an
 14 unpaired t test (***, $P < 0.001$). WT mice (infected and uninfected) include mice
 15 homozygous or heterozygous for functional Batf3. *Right.* Representative histogram
 16 overlay showing the proliferation of PbT-I cells in WT B6 and Batf3-deficient mice. (B)
 17 PbT-I proliferation in DT-treated PbA-infected XCR1-DTRvenus mice. 10^6 CTV-labelled
 18 PbT-I/uGFP cells were transferred into WT or XCR1-DTRvenus mice one day before
 19 infection with 10^4 PbA iRBC. Mice were treated with 500 μ g DT i.p. on days -1, 0, 1 and
 20 2 and were killed on day 5 after infection. *Left.* Numbers of divided PbT-I cells in the
 21 spleen. Data were pooled from 2 independent experiments. For statistical analysis, data
 22 were log transformed and PbT-I proliferation in infected WT vs XCR1-DTRvenus groups
 23 was compared using an unpaired t test (***, $P < 0.001$). *Right.* Representative histograms

1 showing PbT-I cell division. In A and B, each data point represents the number of
2 proliferating PbT-I cells per spleen and the error bars represent SEM. (C) ECM
3 development in Batf3-deficient mice. WT B6 (dotted line) or Batf3-deficient (solid line)
4 mice were infected with 10^4 PbA-iRBC and the development of ECM monitored. The
5 shaded area on the graph represents the period in which mice developed ECM. The
6 difference in the survival between the WT and the Batf3-deficient mice is statistically
7 significant (***, $P < 0.001$) as determined by the Log-Rank test. Data were pooled from 2
8 independent experiments including 15 WT and 7 Batf3-deficient mice. (D) ECM
9 development in XCR1-DTRvenus mice treated with DT during the early stages of the
10 infection. Onset of ECM was monitored in WT (dotted line) and XCR1-DTRvenus (solid
11 line) mice infected with 10^4 PbA-iRBC and treated with 500 μ g DT on days -1, 0, 1 and 2
12 p.i. Curves were statistically compared using a Log-rank test (***, $P < 0.0001$). Data were
13 pooled from 2 independent experiments including 10 WT and 15 XCR1-DTRvenus mice.
14 (E) ECM development in PbA-infected Xcr1-DTRvenus mice treated with DT in a late
15 stage of the infection. The development of ECM was monitored in WT (dotted line) and
16 XCR1-DTRvenus (solid line) mice infected with 10^4 PbA-iRBC and treated with 500 μ g
17 DT on days 5, 6, 7 and 8 p.i. Curves were statistically compared using a Log-rank test (*,
18 $P = 0.0159$). Data were pooled from 2 independent experiments including 10 WT and 14
19 XCR1-DTRvenus mice.

20

21 **Fig 8. PbT-I priming during blood stage PbA is enhanced by CD4⁺ T cell help via**
22 **CD40-CD40L interactions.** (A) CD4⁺ T cell help is required for PbT-I proliferation. 10^6
23 CTV-labelled PbT-I/uGFP cells were transferred into WT or MHC II-deficient (MHC II

1 KO) mice one day before infection with 10^4 PbA iRBC. Numbers of divided PbT-I cells
2 were assessed on day 5 after infection. *Left*. Numbers of divided PbT-I cells in the spleen.
3 *Right*. Representative histograms showing PbT-I cell division. (B) CD4⁺ T cell help is
4 required independently of the starting number of PbT-I cells. 10^6 , 10^5 or 10^4 CTV-labelled
5 PbT-I/uGFP cells were transferred into WT (black circles) or MHC II-deficient (white
6 circles) mice, which were infected with 10^4 PbA iRBC one day later. Numbers of divided
7 PbT-I cells in the spleens were assessed on day 5 after infection. (C) B6 mice were depleted
8 of CD4 T cells by the injection of 100 μ g GK1.5 antibody on days -7 and -4 after infection
9 or left untreated and received 10^6 PbT-I cells one day before infection with 10^6 , 10^5 or 10^4
10 PbA iRBC. Numbers of divided PbT-I cells were estimated on day 5 after infection. Grey:
11 PbT-I numbers in uninfected WT mice. Data were log transformed and analysed using an
12 unpaired t-test (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant). Data were pooled
13 from 2-4 independent experiments. (D) PbT-I proliferation after PbA infection is decreased
14 in CD40L-deficient (CD40L KO) and CD40-deficient (CD40 KO) mice. WT, CD40-
15 deficient or CD40L-deficient mice received 10^6 CTV-labelled PbT-I/uGFP cells and were
16 infected with 10^4 PbA iRBC one day later. Numbers of divided PbT-I cells were assessed
17 on day 5 after infection. *Left*. Numbers of divided PbT-I cells in the spleen. *Right*.
18 Representative histograms showing PbT-I cell division. (E) PbT-II cells restored PbT-I
19 proliferation in PbA-infected CD40L-deficient mice. WT or CD40L-deficient mice were
20 adoptively transferred with 10^6 CTV-labelled PbT-I cells and some mice also received 10^6
21 CTV-labelled PbT-II cells. One day later, mice were infected with 10^4 PbA iRBC.
22 Numbers of divided PbT-I cells were assessed on day 5 after infection. *Left*. Numbers of
23 divided PbT-I cells in the spleen. *Right*. Representative histograms showing PbT-I cell

1 division. Data were log transformed and analysed using One way ANOVA and Tukey's
2 multiple comparisons test (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant). Data
3 were pooled from 2-3 independent experiments. (F) PbT-II cells accelerate ECM
4 development in CD40L-deficient mice. CD40L-deficient mice received 10^6 PbT-II cells
5 (black squares, solid line) or no cells (white squares, solid line) and, together with WT
6 control mice (black circles, dotted line), were infected with 10^4 PbA iRBC one day later.
7 Mice were then monitored for development of ECM. Survival curves of CD40L-deficient
8 mice with or without PbT-II cells were statistically compared using a Log-rank test (**,
9 $P = 0.0011$). Data were pooled from 2 independent experiments including 13 WT, 7 CD40L-
10 deficient mice that received PbT-II cells and 8 CD40L-deficient mice that did not receive
11 PbT-II cells.

12

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Figure 1

Figure 2

Figure 3

Figure 4

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Figure 5

Figure 6

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Figure 8