Early $CD44^{hi}CD4^{+}$ and $CD44^{hi}CD8^{+}$ T cell numbers and the absence of mannose-rich glycoconjugates determine the protective outcome of anti-leishmanial immunity

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SUMMARY

Vaccination remains the best hope for control of all forms of leishmaniasis, and the development of a safe and effective vaccine is a critical global public-health priority. Our previous work showed that immunization with non-persistent phosphomannomutase-deficient (ΔPMM) *Leishmania major* parasites confers significant protection in susceptible BALB/c mice due to increased T-cell numbers and suppression of IL-10 and IL-13 early during infection. Here, we complemented the ΔPMM *L. major* parasites with human PMM2 to determine whether we could further improve the protection. Complemented ΔPMM parasites have restored glycoconjugate biosynthesis, while retaining avirulence of the parental knockout strain. Immunization with hPMM2 add-back parasites showed similar Th1/Th2 cytokine profiles to that observed in ΔPMM-vaccinated mice. However, the numbers of the activated $CD4^{+}CD44^{hi}$ and $CD8^{+}CD44^{hi}$ T cells recruited to the draining lymph nodes early after *Leishmania* infection were reduced, leading to decreased protection following hPMM2-immunization. Thus, the magnitude of T-cell responses early in the infection and the absence of mannose-rich glycoconjugates determine the protective outcome of anti-leishmanial immunity.

Key words: *Leishmania*, vaccine, phosphomannomutase, glycoconjugate.

INTRODUCTION

Leishmaniasis is a disease that ranges in severity from skin lesions to serious disfigurement and fatal systemic infection. WHO estimates that there are 2 million new cases of leishmaniasis each year, 12 million people are currently infected worldwide, and leishmaniasis threatens 350 million people in 88 countries. Therefore, vaccination remains the best hope for control of all forms of the disease, and the development of a safe and effective vaccine is a critical global public-health priority. However, there are no vaccines against leishmaniasis, and chemotherapy is the only means of treatment (Kedzierski *et al*. 2006*b*). The subunit vaccines tested so far did not lead to development of long-term immunity, and the lack of protection has been linked to the requirement for live, persistent parasites maintaining effector T-cell immunity (Uzonna *et al*. 2001). Thus, the live-attenuated vaccine provides an appealing alternative. However, it appears that parasitic persistence is not a desirable feature of a live-attenuated vaccine as evidenced by *L. major* parasites lacking the *lpg2* gene. Although these mutant parasites persist in mice without causing the disease and trigger protective response against virulent challenge (Spath *et al*. 2003; Uzonna *et al*. 2004), they regain their ability to cause infection over time (Spath *et al*. 2004), making them unsuitable as the vaccine. Thus, the non-persistent parasites capable of inducing potent protective anti-leishmanial immunity are of interest as potential vaccine candidates.

We have recently shown that immunization with non-persistent phosphomannomutase-deficient (ΔPMM) parasites confers protection in highly susceptible BALB/c mice (Kedzierski *et al*. 2008). The protection was associated with the increased magnitude of T-cell responses in immunized animals and ability to suppress production of IL-10 and IL-13 early during infection, thus affecting the outcome of the disease in favour of the host. Non-persistence of ΔPMM parasites was a consequence of a deletion of the *pmm* gene, which abrogated biosynthesis of all mannose-rich glycoconjugates, including lipophosphoglycan (LPG), proteophoshpoglycan (PPG) and Parasite Surface Antigen-2 (PSA-2), molecules that are essential to parasite virulence and survival (Garami *et al*. 2001). However, it is possible that deletion of PMM limits the protective anti-leishmanial immunity as strong responses might be directed towards the surface glycoconjugates. Thus, in the present study we set out to determine whether

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ΔPMM parasites complemented with human phosphomannomutase 2 (hPMM2) would still remain non-persistent and avirulent, but elicit even more potent anti-leishmanial immunity and a better protective outcome than that seen following ΔPMM parasite immunization. To address this question, we generated *L. major* ΔPMM parasites complemented with hPMM2 that was expressed either episomally (hPMM2epi) or integrated into ribosomal DNA locus (hPMM2int). Our data show that these add-back parasites express hPMM2 and have restored glycoconjugate biosynthesis (Kedzierski *et al*. 2006*a*), but remain avirulent *in vivo*. Although the Th1/Th2 cytokine profile in hPMM2 add-back vaccinated mice was similar to that observed in ΔPMM vaccinated mice, the numbers of the activated $CD4^+$ $CD44^{hi}$ and $CD8^+CD44^{hi}$ T cells recruited to the draining lymph node early after *Leishmania* infection was reduced. This led to a total lack of protection by immunization with either hPMM2epi or hPMM2int parasites as assessed by lesion scores and parasite burdens. Thus, our study suggests that the magnitude of T-cell responses early in the infection and the absence of mannose-rich glycoconjugates determine the protective outcome of anti-leishmanial immunity.

MATERIALS AND METHODS

Mice, vaccinations and Leishmania *infection*

BALB/c mice at 6–8 weeks were bred at the Institute's animal facility. Animal experiments followed the NHMRC Code of Practice for the Care and Use of Animals for Scientific Purposes guidelines and have been approved by the Institute's Animal Ethics Committee.

L. major MHOM/IL/80/Friedlin, ΔPMM and hPMM2 add-back parasites were maintained at 26 °C in M199 medium supplemented with 10% (v/v) heat inactivated FBS (Trace Biosciences).

To test for parasite virulence naïve BALB/c and nude (athymic) mice at 8 weeks were inoculated subcutaneously/intradermally at the base of the tail with 10^6 parasites (various strains) and lesion development was monitored on weekly bases. Naïve BALB/c mice at 8 weeks were vaccinated intraperitoneally (i.p.) with 5×10^7 hPMM2 add-back or ΔPMM parasites in PBS 4 weeks prior to challenge. Mice were challenged by intradermal injection of 10^6 virulent *L. major* at the base of the tail and lesion development was monitored weekly using a semi-quantitative scoring system (Mitchell *et al*. 1980; Mitchell, 1983). The following scoring was employed: 0 = no lesion or minor injection or healing scar, 1 = small swelling, 2 = large swelling or lesion less than 5 mm in diameter, 3 = lesion 5–10 mm in diameter, 4 = lesion greater than 10 mm in diameter and/or metastasis. Data are expressed as the arithmetic mean ± standard error of the lesion score for the group of mice.

Generation of mutant and add-back parasites

L. major ΔPMM and hPMM2epi parasites were generated as previously described (Kedzierski *et al*. 2006*a*). For the hPMM2 integrated gene add-back, the human pmm2 ORF was amplified from Clone 3611382 NIH Mammalian Gene Collection (Invitrogen) using primers p369 (TCCCCCGGGATGGCAGCGCCTGGCCCAGCG) and p370 (TGCTCTAGATCAGGAGAACAGCAGTTCAC) and cloned into the pIR1SAT vector (a gift from Professor Stephen Beverley, Washington University School of Medicine, St Louis, MO, USA). The linear *SwaI* construct was transfected into *L. major* Friedlin ΔPMM promastigotes and transfectants were selected by growth in medium containing 100 μg/ml of nourseothricin (Jena Bioscience, Germany).

Tissue sampling and cell preparation

Spleens and draining (inguinal) lymph nodes (dLN) were recovered from mice at 1, 2 and 6 weeks following *Leishmania* infection. Spleens and LN were passed through 40 μm sieves to generate a single cell suspension. Parasite burdens were determined by limited dilution analysis (Titus *et al*. 1985). Statistical analyses were performed using one-way ANOVA test with Dunnett's post-test comparing immunized groups to the control group.

Cytokine analyses

Cytokine levels were assessed by capture ELISA and ICS as previously described (Kedzierski *et al*. 2008). Briefly, splenic lymphocytes and dLN cells were resuspended in C-RPMI (10% FBS, 2-mercaptoethanol, antibiotics and L-glutamine), plated in 48-well plates (Costar, NY, USA) in the presence of 4×10^6 irradiated (3000 rad) splenocytes and stimulated for 3 days at 37 °C with soluble *Leishmania* antigen (SLA). For ELISA, culture supernatants were collected following stimulation and added in duplicates at appropriate dilutions to U-bottom 96-well plates (Thermo Labsystems, MA, USA) coated with capture antibodies against IL-4, IL-10, IL-12 (p40/p70) and IFN-γ (BD Pharmingen, CA, USA). Following overnight incubation at 4 °C and washing, biotin anti-mouse IL-4, IL-10, IL-12 (p40/p70) and IFN-γ antibodies were added, followed by incubation with streptavidin-horseradish peroxidase (1 : 5000). Plates were developed with ABTS in 0·1 M citric acid, pH 4·2. Absorbance values were read at 405 nm. IL-13 ELISA was performed using Mouse IL-13 DuoSet ELISA kit (R&D Systems, MN,

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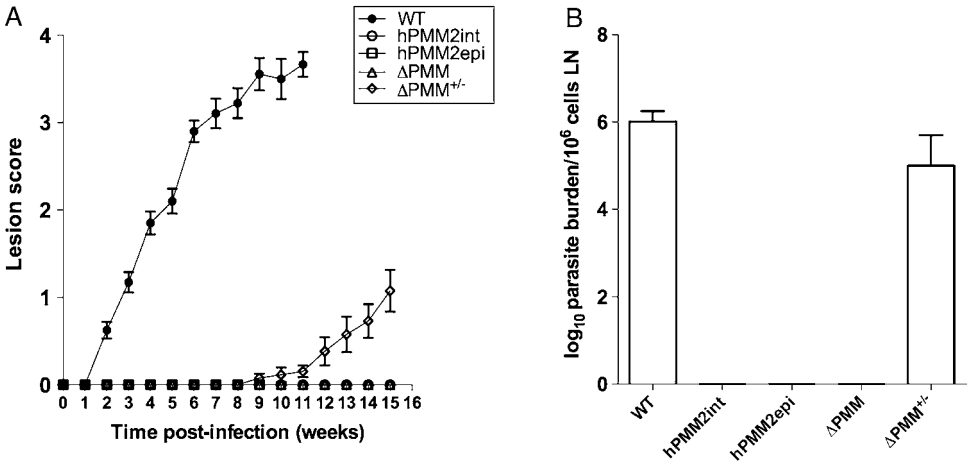

Fig. 1. hPMM2-complemented *Leishmania major* ΔPMM parasites are avirulent *in vivo*. (A) Lesion progression was monitored over a period of 15 weeks. Mean lesion scores (wild type, $n=20$; hPMM2epi, $n=8$, hPMM2int, $n=12$, $\Delta PMM^{+/-}$, $n=12$; ΔPMM, $n=12$) were plotted. Error bars represent the S.E.M. (B) dLN were harvested at 2 months post-infection and parasite burdens were estimated by limited dilution method. Representative data for 2 mice per group are plotted. Error bars represent the S.E.M.

USA). For ICS, cultured cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 2 μg/ml brefeldin A for 4 h at 37 °C. Cells were stained for surface markers CD4-PE, CD8-PerCP and CD44-APC or FITC, fixed and permeabilized. Cells were stained for either IFN-γ-FITC or IL-4-APC (BD Pharmingen). Cell fluorescence was analysed by flow cytometry using a FACSCalibur flow cytometer, data were analysed using BD CellQuest Pro software. Statistical analyses were performed using one-way ANOVA test with Dunnett's post-test comparing immunized groups to the control group.

RESULTS

Human PMM2 add-back parasites are avirulent

We have previously demonstrated that hPMM2epi parasites express glycoconjugates on their surface, but do not survive in macrophages *in vitro* and remain avirulent *in vivo*. Our data show that hPMM2 add-backs (both hPMM2epi (Kedzierski *et al*. 2006*a*) and hPMM2int, data not shown) have restored synthesis of glycoconjugates (PPG, LPG and PSA-2), whereas they could not be detected in ΔPMM parasites. The level of expression as assessed by Western blotting with appropriate antibodies was comparable to that observed in wild type (WT) parasites. *In vitro*, add-back parasites do not display any growth abnormalities, but hPMM2epi have growth kinetics similar to ΔPMM parasites rather than WT *L. major* (Kedzierski *et al*. 2006*a*). Here, we tested the ability of hPMM2int add-backs to cause chronic *Leishmania* infection in susceptible BALB/c mice in comparison to WT parasites, hPMM2epi add-backs, ΔPMM parasites and $PMM^{+/-}$ single knockouts. Following intradermal injection of mice with either 10^6 hPMM2epi or hPMM2int add-back parasites at the base of the tail, no lesion development was observed after 15 weeks (Fig. 1A), or subsequent 3 months (data not shown). Furthermore, parasites could not be recovered from dLN at 2 months post-infection (Fig. 1B), confirming the avirulent nature of hPMM2-complemented parasites. Similarly, injection of hPMM2epi and hPMM2int parasites into the nude mice led to a lack of any lesion development during a period of 8 months (data not shown).

Immunization with PMM2 add-back parasites leads to suppression of IL-10 and IL-13 production early during infection

Immunization with non-persistent ΔPMM parasites led to a strong anti-leishmanial protection associated with increased T-cell numbers and ability to suppress production of IL-10 and IL-13 early during infection (Kedzierski *et al*. 2008). To assess the immune responses in mice immunized with live hPMM2-complemented parasites, we determined the levels of Th1-type cytokines (IFN-γ and IL-12) associated with protection (Fig. 2) and Th2-type cytokines (IL-4, IL-10 and IL-13) linked to suppression of protective immunity (Fig. 3). Cytokine production was assessed in spleen and dLN cells after 3 days *ex vivo* stimulation with soluble *Leishmania* antigen (SLA) by intracellular cytokine staining (ICS) and capture ELISA.

As reported previously for ΔPMM immunization (Kedzierski *et al*. 2008), no IL-4 secretion and no significant difference in the numbers of either $CD44^{hi}CD4^{+}$ or $CD44^{hi}CD8^{+}$ T cells producing IFN-γ could be detected by ICS in any of the groups

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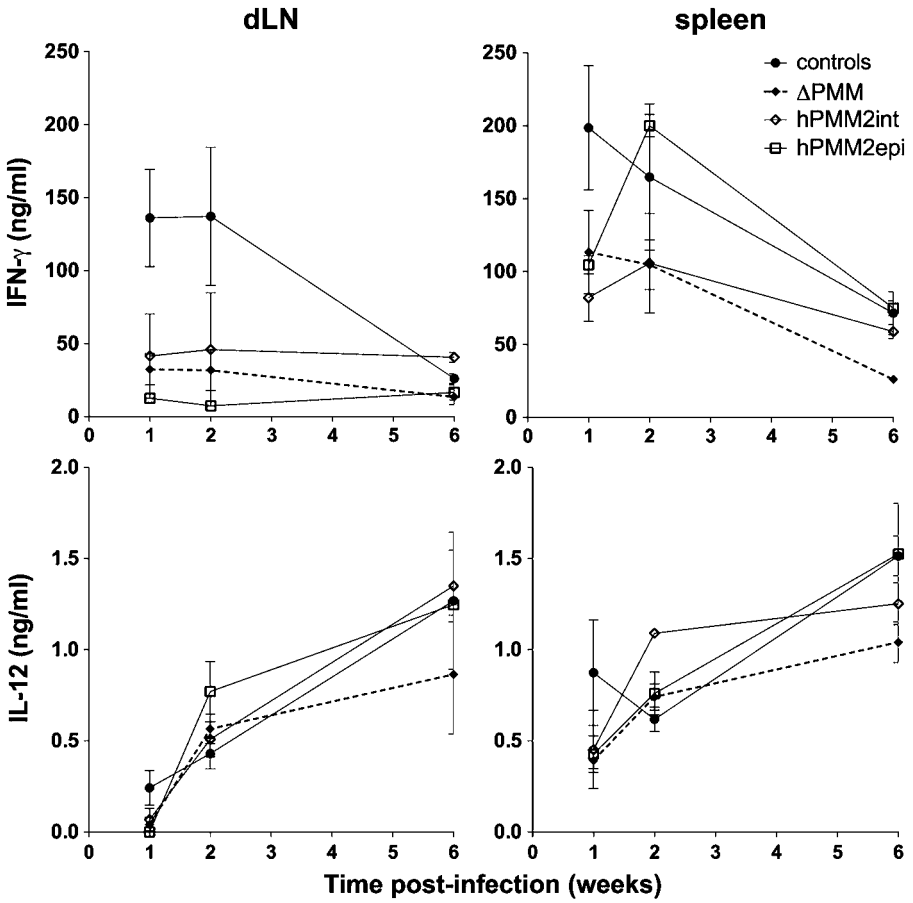

Fig. 2. ELISA analysis of Th1-type cytokine production by splenocytes and dLN cells from non-immunized controls and mice immunized with live hPMM2 add-back and ΔPMM parasites. At 1, 2 and 6 weeks post-challenge spleens and dLN cells were stimulated with SLA. Following 72 h stimulation at 37 °C, supernatants were collected and analysed for secretion of IFN-γ and IL-12 by ELISA. Results are from independent experiments for each time-point, mean data plotted ($n=3$, in duplicates), error bars represent the S.E.M.

at any time-point in either spleen or dLN (data not shown). Consistent with the ICS data, low levels of IL-4 secretion were detected by ELISA, with no differences found between the groups at different time-points (Fig. 3). Similarly, no differences in either IFN-γ or IL-12 production were observed between the control or immunized groups (Fig. 2).

However, the significant differences were observed early after *Leishmania* infection (week 1) in production of IL-10 and IL-13 in both the spleen and dLN of control mice compared to hPMM2 add-back immunized animals. An average of $3{\cdot}18 \pm 0{\cdot}46$ ng/ml of IL-10 was detected in the cultured splenocytes of non-immunized animals compared to $0{\cdot}76 \pm 0{\cdot}14$ ng/ml for hPMM2epi (P value in a range 0·001–0·01) and $0{\cdot}99 \pm 0{\cdot}02$ ng/ml for hPMM2int (P value in a range 0·001–0·01) vaccinated mice. Similar differences were observed in dLN culture supernatants with controls showing $5{\cdot}0 \pm 0{\cdot}27$ ng/ml of IL-10 compared to an average of $0{\cdot}36 \pm 0{\cdot}36$ ng/ml for hPMM2int ($P<0{\cdot}001$), while in hPMM2epi immunized mice IL-10 levels were below the detection level. An average of $12{\cdot}8 \pm 0{\cdot}69$ ng/ml of IL-13 was detected for the non-immunized animals compared to $3{\cdot}26 \pm 0{\cdot}02$ ng/ml for hPMM2epi immunized mice ($P<0{\cdot}001$) and $4{\cdot}94 \pm 0{\cdot}54$ ng/ml for hPMM2int immunized mice ($P=0{\cdot}004$). In dLN culture supernatants control mice produced $13{\cdot}0 \pm 0{\cdot}74$ ng/ml *vs* 0·12 ng/ml ($P<0{\cdot}001$) and $1{\cdot}53 \pm 1{\cdot}29$ ng/ml ($P<0{\cdot}001$) for hPMM2epi and hPMM2int immunized mice, respectively.

No significant differences in cytokine production were found between hPMM2epi and hPMM2int immunized mice. Cytokine profiles of both groups were comparable to ΔPMM-immunized mice (Kedzierski *et al.* 2008) (Figs 2 and 3), which rendered significant protection and anti-leishmanial immunity.

Immunization with hPMM2 add-back parasites does not lead to the recruitment of the activated $CD44^{hi}CD4^{+}$ and $CD44^{hi}CD8^{+}$ T cells to the draining lymph node

Immunization with ΔPMM parasites leads to expansion of $CD44^{hi}CD4^{+}$ and $CD44^{hi}CD8^{+}$ T cell numbers and their rapid recruitment into dLN following *Leishmania* infection (Kedzierski *et al.* 2008).

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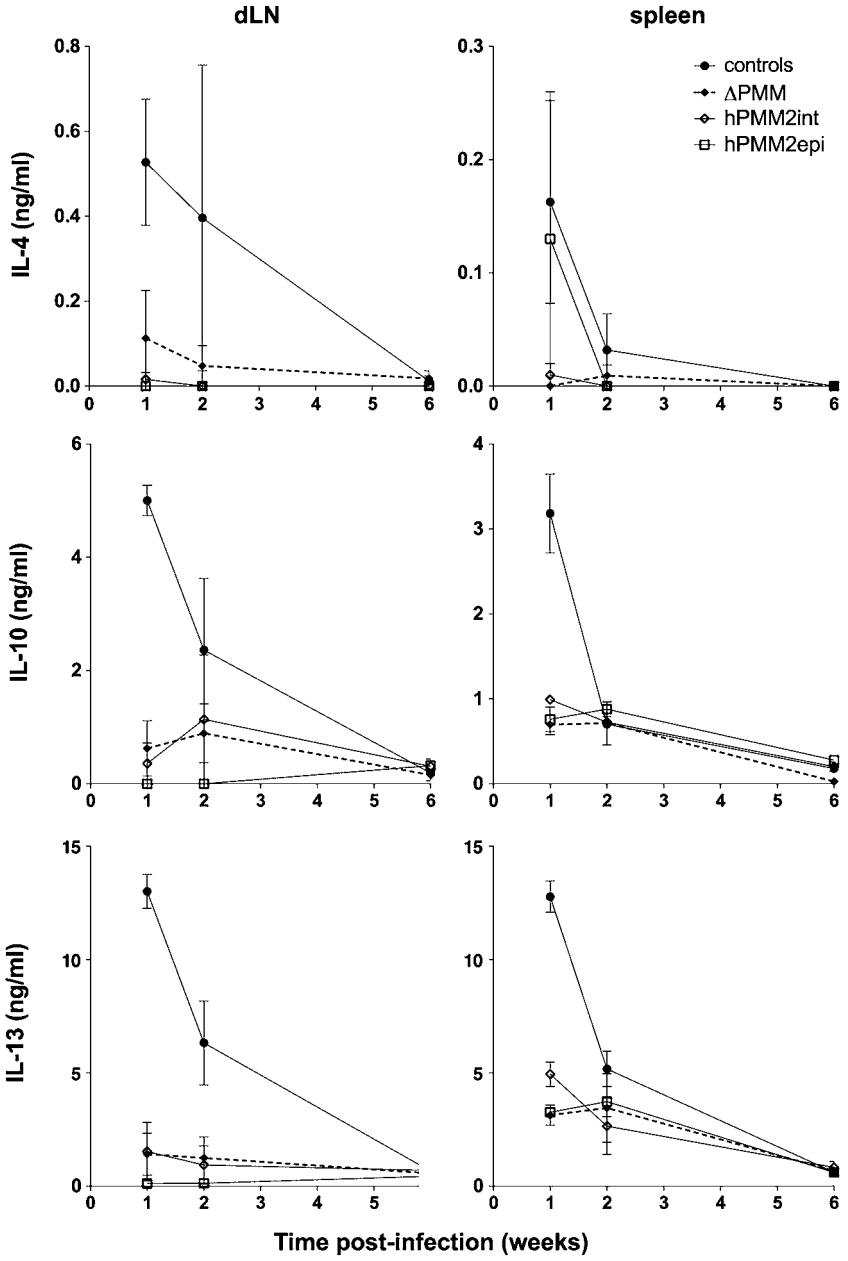

Fig. 3. ELISA analysis of Th2-type cytokine production by splenocytes and dLN cells from non-immunized controls and mice immunized with live hPMM2 add-back and ΔPMM parasites. At 1, 2 and 6 weeks post-challenge spleens and dLN cells were stimulated with SLA. Following 72 h stimulation at 37 °C, supernatants were collected and analysed for secretion of IL-4, IL-10, and IL-13 by ELISA. Results are from independent experiments for each time-point, mean data plotted ($n=3$, in duplicates), error bars represent the S.E.M.

We have assessed the numbers of activated $CD44^{hi}CD8^{+}$ and $CD44^{hi}CD4^{+}$T cells in mice immunized with hPMM2-complemented parasites at week 1 and 2 following the virulent challenge (Fig. 4). There was no significant increase in numbers of recruited $CD44^{hi}CD8^{+}$ and $CD44^{hi}CD4^{+}$ T cells in mice immunized with hPMM2int or hPMM2epi compared to controls, while in mice immunized with ΔPMM parasites we observed an increase in number of recruited $CD44^{hi}CD8^{+}$ (p value in a range 0·001–0·01 *cf.* controls) and $CD44^{hi}CD4^{+}$ T cells. At week 2 post-infection hPMM2int-immunized mice showed no difference in numbers of recruited antigen experienced cells compared to controls, while in hPMM2epi-immunized mice we observed a decrease in the magnitude of T cell responses. Overall, numbers of $CD44^{hi}CD8^{+}$ and $CD44^{hi}CD4^{+}$ T cells in add-back immunized groups were maintained at the same or lower level as in controls, whereas ΔPMM-immunized animals displayed increased numbers of both $CD44^{hi}CD8^{+}$ and $CD44^{hi}CD4^{+}$ T cells in dLN at the early stages of *Leishmania* infection (Fig. 4).

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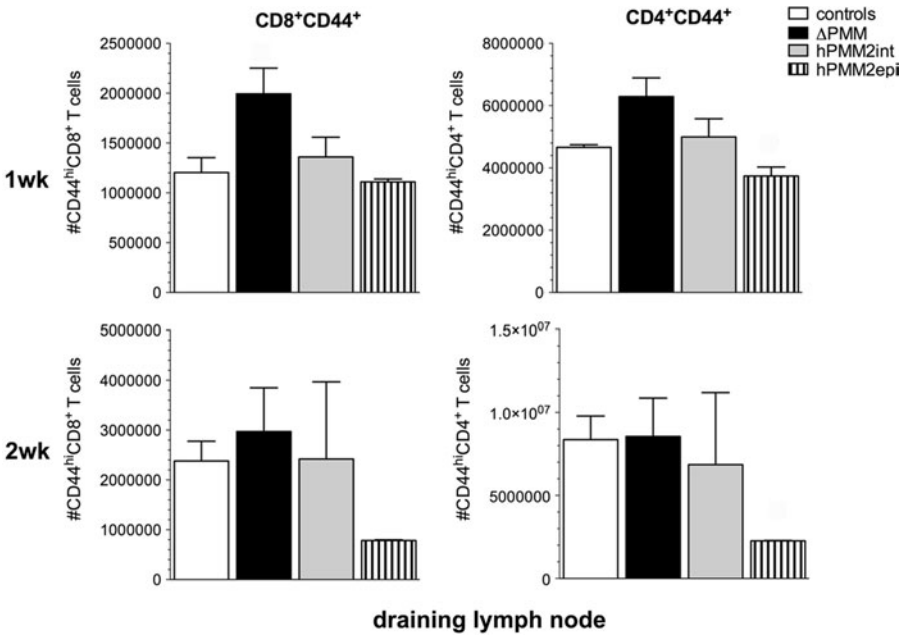

Fig. 4. Numbers of $CD8^+CD44^{hi}$ and $CD4^+CD44^{hi}$ T cells early after *Leishmania major* challenge. Numbers of antigen-experienced $CD44^{hi}CD8^+$ (left panel) and $CD44^{hi}CD4^+$ (right panel) T cells within dLN were calculated based on total cell counts and staining with mAbs against surface CD44 and CD4 or CD8. Data are plotted as absolute numbers of $CD44^{hi}CD8^+$ and $CD44^{hi}CD4^+$ detected in draining lymph nodes by FACS and represent pooled dLN for each mouse and a mean value from 3 mice per group. Results are from independent experiments for each time-point. Error bars represent the S.E.M.

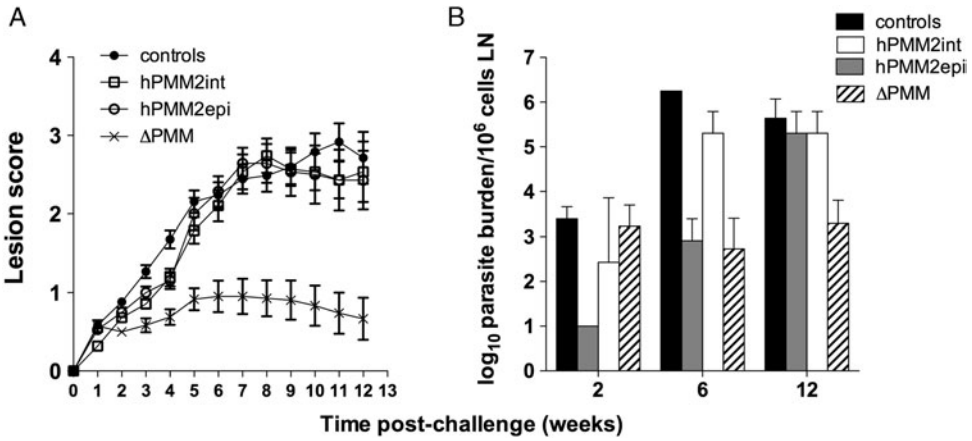

Fig. 5. Immunization with live, hPMM2-complemented ΔPMM *Leishmania major* parasites does not provide protection against virulent challenge. (A) Mice were vaccinated i.p. with 5×10^7 live hPMM2int ($n=29$) or hPMM2epi ($n=14$) parasites 4 weeks prior to infection and challenged along with naïve ($n=36$) and ΔPMM immunized ($n=22$) mice with 10^6 *L. major* Friedlin parasites at the base of the tail. Lesion scores were monitored for 12 weeks, data plotted for 2 independent experiments, error bars represent the S.E.M. (B) Parasite burdens in hPMM2int and hPMM2epi vaccinated and non-vaccinated mice. dLN were harvested at different time-points following challenge and parasite burdens estimated by limited dilution method. Pooled data from 2 mice are plotted, error bars represent the S.E.M. Representative data from ΔPMM vaccinated mice included for comparison ($n=6$ for week 2 and $n=11$ for week 6 and 11).

Vaccination with hPMM2 add-back parasites does not protect mice against virulent L. major *challenge*

To investigate whether live hPMM2 add-back parasites can provide protection against *L. major* infection, groups of 20 susceptible BALB/c mice were immunized i.p. with 5×10^7 hPMM2 add-back or ΔPMM parasites at 4 weeks prior to challenge. Naïve controls and vaccinated mice were subsequently challenged intradermally with 10^6 virulent *L. major* Friedlin parasites. The majority of control mice developed progressive infection and were sacrificed at 12 weeks post-challenge. As predicted from the early $CD44^{hi}CD4^+$ and $CD44^{hi}CD8^+$ T cell responses in dLN (Fig. 5), animals immunized with hPMM2 add-back parasites were not able to control

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the infection and developed lesions identical to those observed in unvaccinated controls (Fig. 5A).

Mice from all the vaccinated groups were sacrificed at 2, 6 and 12 weeks post-challenge and parasite burdens in the dLN were estimated by limiting dilution analysis. In agreement with lesion size, vaccinated mice showed no difference in parasitic load compared to controls throughout the trial, despite an early trend indicating smaller parasite burdens in vaccinated animals (Fig. 3B). Thus, despite the favourable IFN-γ/IL-10 ratio in spleen and dLN of mice immunized with hPMM2 add-back parasites, these animals were not protected against virulent challenge, consistent with lower numbers of the activated *Leishmania*-specific $CD44^{hi}CD8^{+}$ T cells and $CD44^{hi}CD4^{+}$ T cells compared to those achieved after protective ΔPMM immunization.

DISCUSSION

Our study assessed protective efficacy of *L. major* ΔPMM parasites complemented with hPMM2, and showed that mice were not protected following immunization with live add-back parasites. This is in contrast with our previous study showing that ΔPMM parasites were able to trigger protective anti-leishmanial responses in susceptible BALB/c mice (Kedzierski *et al.* 2008). Paradoxically, hPMM2 complement parasites have restored surface glycoconjugate biosynthesis, yet remain avirulent in macrophages *in vitro* and in mice *in vivo*, behaviour reminiscent of ΔPMM parasites.

Leishmanial PMM shares a high degree of similarity with its human isoforms, PMM1 and PMM2 (Kedzierski *et al.* 2006*a*), therefore it was not surprising that hPMM2 was able to functionally complement the knockout parasites. However, the reason for their avirulence remains to be elucidated. Under culture conditions, hPMM2epi parasites maintain the episome due to the drug pressure, which can then be lost *in vivo* rendering the parasites virtually identical to ΔPMM mutants. However, in case of the hPMM2int add-back, the *pmm2* has been integrated into the chromosomal locus of ΔPMM parasites and its expression is independent of drug selection. We have demonstrated that one copy of indigenous *pmm* gene is sufficient for the parasite to remain virulent, albeit with a delayed phenotype. The avirulent nature of hPMM2-complemented parasites opened an interesting possibility to explore their protective potential as a live-attenuated vaccine. Their intracellular killing and degradation should provide a full complement of peptides for presentation and all molecules that are essential for parasite survival and virulence should become available for presentation. Due to an as yet unidentified defect these molecules are not able to restore parasite virulence, hence they might not be able to modulate macrophage function and cytokine responses as reported for the wild-type parasites (Bogdan and Rollinghoff, 1998). In principle, these parasites should trigger a better immune response that leads to a better protective outcome than that observed following immunization with ΔPMM parasites.

Unexpectedly, the complementation with hPMM2 led to the loss of protective capacity of ΔPMM parasites. This was due, at least in part, to reduced numbers of $CD44^{hi}CD8^{+}$ T cells and $CD44^{hi}CD4^{+}$ T cells in dLN early after *Leishmania* immunization. The cytokine profile in add-back immunized animals did not differ significantly between the two groups nor was it much different from that observed following ΔPMM immunization (Kedzierski *et al.* 2008). We have not observed a significant imbalance in cytokine profile indicating skewing towards the deleterious Th2-type response. Strikingly though, these animals showed an impaired expansion of dLN following virulent challenge, which suggests that decreased magnitude of the T-cell response is responsible for the observed lack of protection. Since the only difference between ΔPMM and ΔPMM + hPMM2 parasites was restoration of mannose-rich glycoconjugate synthesis, our findings suggest that these molecules led to modulation of the immune response following priming with hPMM2-complemented parasites, and prevented recruitment of activated T cells in the dLN. LPG is the most abundant glycoconjugate on the promastigote surface and has been previously shown to modulate several host cell functions (Turco and Descoteaux, 1992). LPG can inhibit the migratory activity of Langerhans cells (LC) and reduce their efflux from the site of infection to dLN (Ponte-Sucre *et al.* 2001). Migration and maturation of LC have been postulated to be critical for initiation of T-cell responses and antigen presentation, since the infected macrophages are deficient in expression of their co-stimulatory molecules necessary for T-cell activation, and have a greatly reduced capacity to present antigens following *Leishmania* infection (Fruth *et al.* 1993; Kaye *et al.* 1994). Hence, it is possible that the glycoconjugates expressed by hPMM2 add-back parasites (including LPG) were sufficient to interfere with the dendritic cell and macrophage functions upon priming with hPMM2 add-backs, and subsequently led to decreased numbers of primed T cells. As a consequence, insufficient numbers of *Leishmania*-specific memory T cells were available for recruitment to dLN following secondary, virulent challenge. Indeed, recent studies demonstrated that dendritic cells are required for draining lymph node expansion (Angeli *et al.* 2006; Webster *et al.* 2006), and that insufficient activation of dendritic cells leads to the impaired dLN expansion following *L. mexicana* infection (Hsu and Scott, 2007).

Our findings highlight the importance of the early T-cell responses for the control of the *Leishmania*

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infection as well as suggesting that vaccination with live-attenuated non-persistent parasites is feasible only in the absence of mannose-rich glycoconjugates abundantly present on the parasite surface. Therefore, our study provides a novel contribution to the understanding of anti-leishmanial immunity important for the rational design of vaccination and/or immunotherapy protocols.

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