

Conditions Influencing the Efficacy of Vaccination with Live Organisms against *Leishmania major* Infection

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Numerous experimental vaccines have been developed with the goal of generating long-term cell-mediated immunity to the obligate intracellular parasite *Leishmania major*, yet inoculation with live, wild-type *L. major* remains the only successful vaccine in humans. We examined the expression of immunity at the site of secondary, low-dose challenge in the ear dermis to determine the kinetics of parasite clearance and the early events associated with the protection conferred by vaccination with live *L. major* organisms in C57BL/6 mice. Particular attention was given to the route of vaccination. We observed that the rapidity, strength, and durability of the memory response following subcutaneous vaccination with live parasites in the footpad are even greater than previously appreciated. Antigen-specific gamma interferon (IFN- γ)-producing T cells infiltrate the secondary site by 1.5 weeks, and viable parasites are cleared as early as 2.5 weeks following rechallenge, followed by a rapid drop in IFN- γ ⁺ CD4⁺ cell numbers in the site. In comparison, intradermal vaccination with live parasites in the ear generates immunity that is delayed in effector cell recruitment to the rechallenge site and in the clearance of parasites from the site. This compromised immunity was associated with a rapid recruitment of interleukin-10 (IL-10)-producing CD4⁺ T cells to the rechallenge site. Treatment with anti-IL-10-receptor or anti-CD25 antibody enhanced early parasite clearance in ear-vaccinated mice, indicating that chronic infection in the skin generates a population of regulatory cells capable of influencing the level of resistance to reinfection. A delicate balance of effector and regulatory T cells may be required to optimize the potency and durability of vaccines against *Leishmaniasis* and other intracellular pathogens.

Currently, vaccines developed for use in humans that reliably induce cell-mediated immunity, thereby providing protection against obligate intracellular pathogens such as *Mycobacterium tuberculosis* and *Leishmania*, have met with limited success (10, 17, 25, 30). In the case of leishmaniasis, immunization with live, virulent *Leishmania major*, resulting in a lesion that heals, is referred to as leishmanization and is the only proven “vaccination” strategy in humans (12, 16, 28). While highly effective, leishmanization has been largely abandoned due to safety concerns, mostly surrounding the development of ulcerating primary lesions that were slow to heal, or, in rare cases, nonhealing lesions (24). In an attempt to emulate the long-lasting cell-mediated immunity conferred by leishmanization without the adverse effects of a primary infection, nonliving protein- or DNA-based vaccines or live vaccines employing recombinant vectors or attenuated parasites have been developed that induce no, or very limited, primary disease (11, 15).

In the mouse model of leishmaniasis, antigen persistence is a critical factor for the maintenance of protective immunity, as both BALB/c and C57BL/6 mice that are manipulated to achieve sterile cure following primary infection with *L. major*

also lose their resistance to reinfection (6, 33). Protective immunity appears to be optimized by the presence of at the time of secondary challenge of both antigen-independent central memory cells and antigen-dependent effector cells (34). The requirement for persisting antigen appears, at least partially, to be responsible for the fact that the *Leishmania*-specific immunity generated by nonliving vaccines, either alone or in conjunction with adjuvants, has the tendency to wane, is nonprotective over the long term, and has failed to match the potency and durability of vaccination with live organisms in both murine and human disease (1, 13, 14, 17, 20, 22, 25, 29–32).

Despite its proven efficacy, the early events associated with the expression of immunity in the rechallenge site and variables, such as route of inoculation, that might optimize or compromise the efficacy of live vaccines have yet to be carefully addressed. The route of inoculation seems especially relevant, as the site of primary infection with *Leishmania* is known to influence the immune response and outcome of disease (3, 5, 9, 23, 26). We were particularly interested to address how the generation of interleukin-10 (IL-10)-producing CD4⁺ CD25⁺ regulatory T (T_{REG}) cells, which have been shown to control parasite persistence in the skin and to compromise the expression of immunity to reinfection (6, 21), might be altered following primary infection/vaccination in different sites.

In the present report we attempt to more fully define the protective secondary immune response generated by the use of live vaccine and the influence of the site of vaccination on this response. We give particular attention to the dynamic interplay between effector T (T_{EFF}) and T_{REG} cells in the secondary challenge site and find that the site of vaccination—subcuta-

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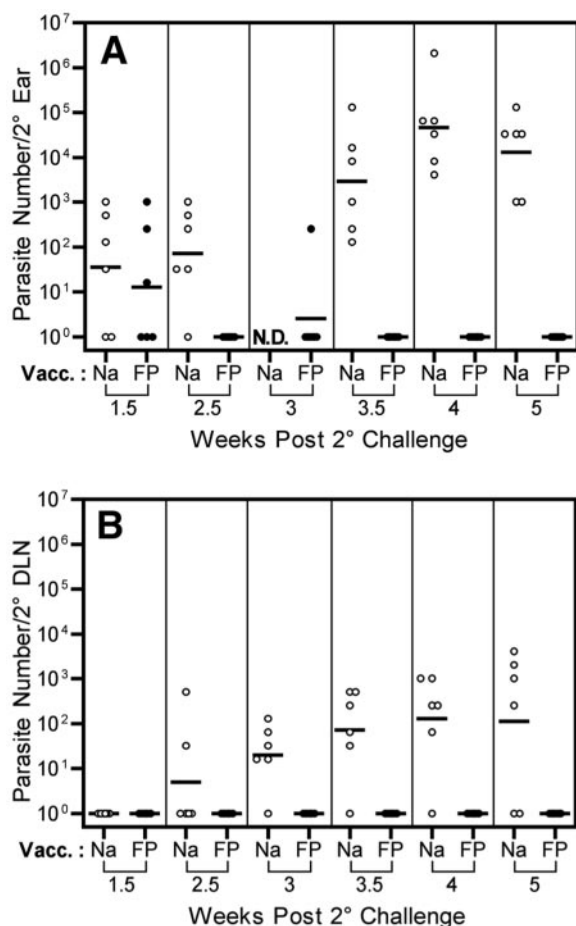


FIG. 1. Protective immunity generated by FP vaccination with live parasites is characterized by the rapid clearance of parasites from a site of secondary challenge. Naive C57BL/6 mice (Na) or mice vaccinated 16 weeks prior in the RFP with 10^4 *L. major* V1 metacyclic promastigotes (FP) were rechallenged, i.d., in both ears with 500 metacyclic promastigotes. Parasite loads were determined in the secondary ear dermis site (A) or DLN (B) at the indicated time points following rechallenge. Results shown represent parasite loads (per ear or DLN) for individual ears or DLNs. Three mice were employed per group per time point. ND, not done.

neous versus intradermal—with live parasites, can significantly influence the cell types recruited to the secondary site, the strength of the secondary immune response, and the efficiency of parasite clearance upon rechallenge.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD) or Taconic (Rockville, MD). All mice were maintained at the National Institute of Allergy and Infectious Diseases animal care facility under pathogen-free conditions.

Parasite preparation and live vaccination protocol. *L. major* clone V1 (MHOM/IL/80/Friedlin) promastigotes were grown at 26°C in medium 199 supplemented (M199/S) with 20% Hi-fetal calf serum (HyClone, Logan, UT), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50 mM HEPES), 5 mg/ml hemin (in 50% triethanolamine), and 1 mg/ml 6-biotin. Infective-stage promastigotes (metacyclics) of *L. major* were isolated from stationary cultures (4- to 5-day-old cultures) by negative selection of infective forms using peanut agglutinin (Vector Laboratories, Inc., Burlingame, CA). Mice were infected in the ear dermis by intradermal (i.d.) injection

using a 29.5-gauge needle in a volume of 10 µl or in the footpad (FP) by subcutaneous (s.c.) injection using a 27.5-gauge needle in a volume of 40 µl. Care was taken during footpad injection to avoid the intramuscular and/or intradermal tissue.

Lymphocyte preparation. The ventral and dorsal sheets of the infected ears were separated, deposited dermal side down in Dulbecco's modified Eagle's medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, and Liberase CI enzyme blend (50 µg/ml; Boehringer Mannheim). Ears were incubated for 2 h at 37°C. Footpads were similarly prepared following removal of the toes and bones. Ear sheets and footpad tissues were ground in a Medimachine (Beckton Dickinson). Retromaxilar (ears) and popliteal (FP) lymph nodes were removed and mechanically dissociated using a pellet pestle in 100 µl of M199/S medium. Tissue homogenates of both infected tissues and draining lymph nodes (DLN) were filtered using a 70-µm-pore-size cell strainer (Falcon Products, Inc. St. Louis, MO).

Estimation of parasite load. Parasite loads in the ear, FP, and DLN were determined as previously described (4). Briefly, recovered lymphocytes were serially diluted in a 96-well flat-bottom microtiter plate containing biphasic medium prepared using 50 µl of NNN medium containing 20% of defibrinated rabbit blood and overlaid with 100 µl of M199/S medium. The number of viable parasites in each ear was determined from the highest dilution at which promastigotes could be grown out after 7 to 10 days of incubation at 26°C.

In vitro recall response and fluorescence-activated cell sorting analysis. Mice were sacrificed and single-cell suspensions from the ear dermis, FP, and local DLN (pooled cells from 3 to 5 mice per time point) were obtained as described above. For the analysis of surface markers and intracytoplasmic staining for gamma interferon (IFN-γ) and IL-10, cells were stimulated with *L. major*-infected murine bone marrow-derived dendritic cells (BMDDC) as a source of antigen for 16 h, at which time brefeldin A was added (10 µg/ml) (7). The cells were cultured for an additional 6 h and then fixed in 4% paraformaldehyde. Prior to staining, cells were incubated with an anti-Fcγ III/II (Pharmlingen) receptor and 10% normal mouse serum (NMS) in phosphate-buffered saline containing 0.1% bovine serum albumin and 0.01% Na₂S₂O₈. Cells were permeabilized and stained for the surface markers T-cell receptor β (145-2 C11; fluorescein isothiocyanate) (Pharmlingen), CD4 (RM4-5; Cychrome, Pharmlingen), and CD25 (PC61; phycoerythrin; Pharmlingen) and for the cytokines IFN-γ (JE56-5H4; Pharmlingen) and IL-10 (JES5-16E3; Pharmlingen) conjugated to R-PE. Incubations were carried out for 30 min on ice. The frequency of CD4⁺ T cells was determined by gating on T-cell receptor β⁺ cells. For each sample 50,000 (see Fig. 2 and 4) or 200,000 (see Fig. 6) cells were analyzed. The data were collected and analyzed using CELLQuest software and a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA).

Measurement of IFN-γ and IL-10 production. For cytokine measurement in culture supernatants, pooled cells from DLN were resuspended in RPMI medium containing fetal bovine serum-penicillin-streptomycin at 6×10^6 cells/ml, and 0.1 ml was plated in triplicate in U-bottom 96-well plates. Cells were incubated at 37°C in 5% CO₂ with uninfected or with *L. major*-infected BMDDC. IFN-γ and IL-10 production in 48-h culture supernatants was quantitated by enzyme-linked immunosorbent assay (ELISA; Endogen, Woburn, MA).

Anti-CD25 and anti-IL-10R treatments. Mice were infected with 10^4 metacyclic promastigotes in the FP or ear, and 10 weeks following infection, ear-infected mice were injected intraperitoneally with 0.5 mg of anti-CD25 (PC61) anti-IL-10 receptor (IL-10R; 1B1.3a) or control antibody (GL113) on days -3, 0, and +3. Antibodies were purified by ammonium sulfate precipitation and ion exchange chromatography. On day 0, mice were challenged in both ears (FP-immunized mice) or the contralateral ear (ear-immunized mice) with 500 to 1,000 metacyclic promastigotes. Parasite loads of ears, footpads, and DLN were determined 3 weeks following challenge.

Statistical analysis. Statistical significance between means of various groups was determined using a two-tailed *t* test for independent samples.

RESULTS

Protective immunity generated by s.c. vaccination with live parasites in the footpad. In order to establish the efficacy of using live vaccine, we immunized C57BL/6 mice with live parasites and assessed the early kinetics of parasite clearance and the immune response at the site of secondary challenge. In initial studies, we employed s.c. primary infection in the FP with 10^4 live *L. major* metacyclic promastigotes, as this inoculum is consistent with the dose used for leishmanization in

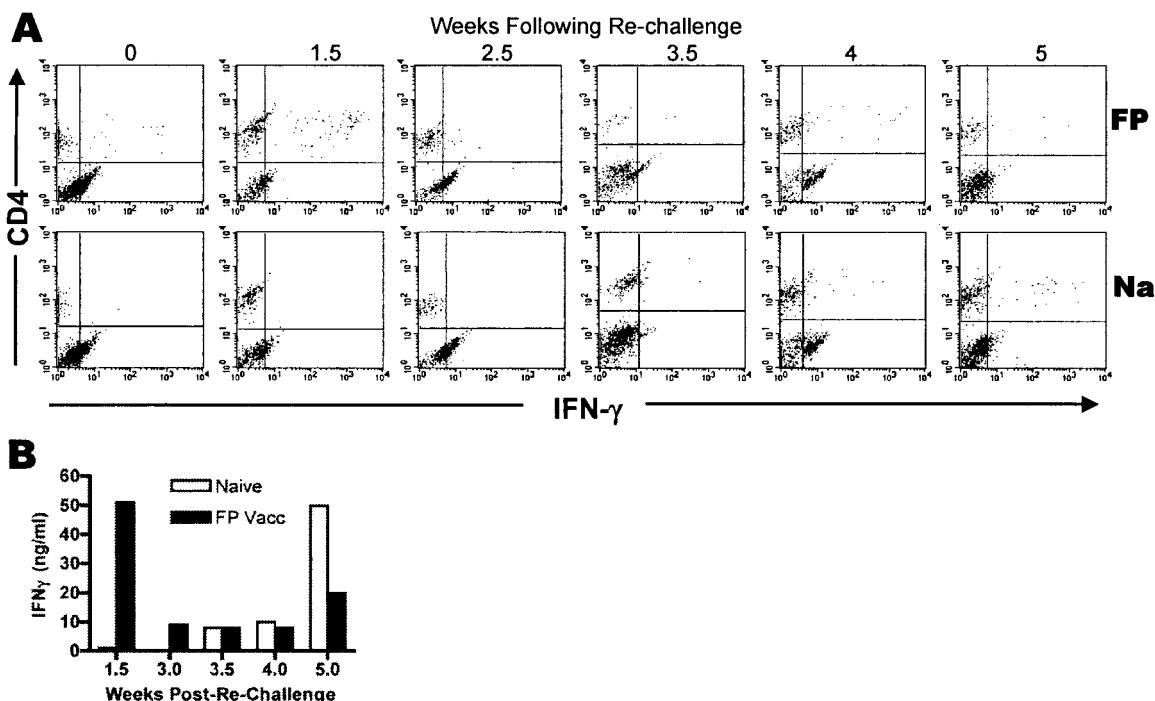


FIG. 2. Protective immunity generated by FP vaccination with live parasites is characterized by the rapid recruitment of IFN- γ -producing CD4⁺ T cells to the site of secondary challenge. Naïve mice (Na) or mice vaccinated 16 weeks prior in the RFP with 10⁴ *L. major* metacyclic promastigotes (FP) were rechallenged in both ears with 500 metacyclic promastigotes. (A) At various time points following rechallenge, cells from the ear dermis were restimulated in vitro with *L. major*-infected dendritic cells and stained for surface markers and intracytoplasmic IFN- γ . (B) IFN- γ production by pooled cells from the DLN of the secondary site following in vitro restimulation with *L. major*-infected BMDDC as determined by ELISA.

human trials. The FP was chosen in order to spare both ear dermal sites for secondary challenge. Intradermal reinfection in each ear with 500 to 1,000 *L. major* metacyclics was used to more closely reproduce the tissue site and dose of natural challenge. Secondary challenge was performed 16 weeks after vaccination with live parasites as a stringent test of vaccine durability. At this time point, the primary lesions have completely resolved, and a low number of parasites remain in the primary site (data not shown). Vaccinated and naïve control mice harbored similar numbers of viable parasites 1.5 weeks after challenge (Fig. 1A). This number steadily increased in naïve mice, attaining a geometric mean of 4.6×10^4 parasites per ear in the fourth week. In contrast, parasites were sharply controlled in FP-immunized mice, where, with the exception of only one ear at 3 weeks, viable parasites were completely cleared by 2.5 weeks postinfection. We also analyzed the DLN for parasite dissemination from the injection site. Parasites were detectable in the DLN of naïve mice as early as 2.5 weeks following challenge (Fig. 1B), increasing in numbers in the following weeks to a geometric mean of 144/DLN in the fourth week of infection. In contrast, the dissemination of parasites from the ear to the DLN of FP-immunized mice was not detectable at any time following secondary challenge. These data indicate that the immune state generated following the use of live vaccine is extremely potent, leading to a rapid and efficient clearance of parasites from the site of reinfection.

Shortly after reinfection (1.5 weeks), the secondary site in FP-vaccinated mice was characterized by the infiltration of large numbers of lymphocytes into the ear dermis (1.8×10^5 /

ear). This infiltrate included a high proportion of antigen (Ag)-specific IFN- γ -producing CD4⁺ T lymphocytes, representing 31% of total CD4⁺ T cells (Fig. 2A). Over the course of parasite clearance from the injection site, the number of IFN- γ -producing CD4⁺ lymphocytes waned, but they did not entirely disappear. Similarly, IFN- γ production by DLN cells from FP-vaccinated mice peaked sharply at 1.5 weeks following rechallenge and waned quickly thereafter (Fig. 2B). In naïve mice the immune response developed much more slowly, with the appearance of infiltrating IFN- γ -producing CD4⁺ lymphocytes delayed until 4 weeks, eventually representing 28% of total CD4⁺ T cells. These findings were also reflected in the lymph nodes of naïve mice, where IFN- γ production was first detected in low concentrations at 3.5 weeks following rechallenge and increasing at 5 weeks, the last time point analyzed.

Comparison of protective immunity generated by s.c. versus i.d. vaccination with live parasites. We were struck in these studies by the dramatic protection achieved by FP vaccination, especially in comparison with a number of previous studies involving vaccination with live parasites in the ear dermis and secondary challenge in the contralateral ear, in which parasite clearance did not appear to be nearly so efficient (21, 22). To directly address whether the site of primary infection can influence the efficacy of the use of live vaccine, mice were vaccinated with 10⁴ live metacyclic *L. major* promastigotes, either i.d. in the right ear or s.c. in the right FP (RFP) and challenged 7 weeks later in the contralateral ear with 10³ metacyclics. At 1.5 weeks postchallenge there was no significant difference in the ear parasite loads between the ear dermis-immunized mice

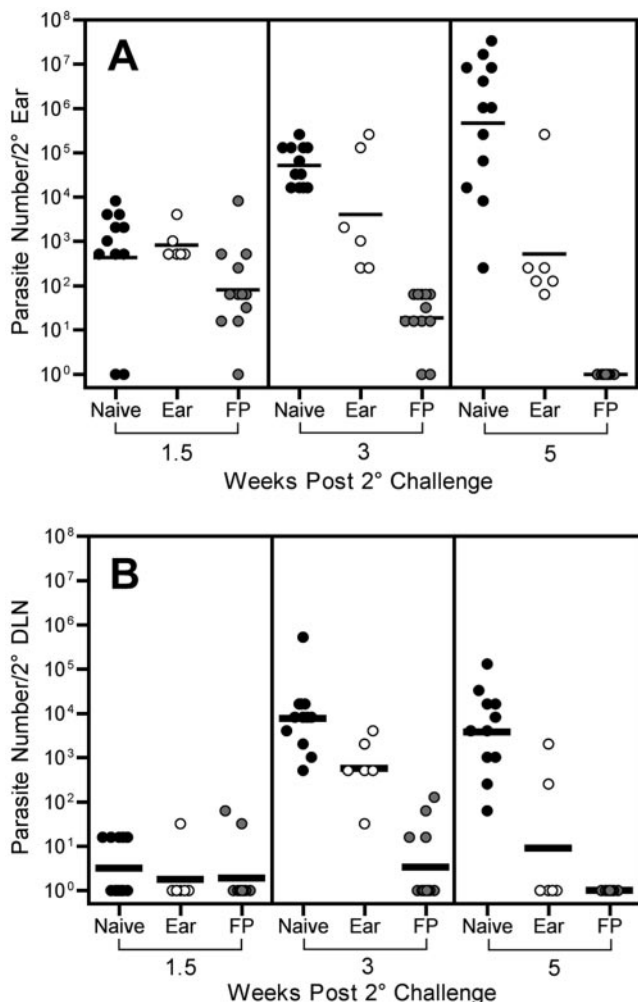


FIG. 3. Parasite clearance from a site of secondary challenge is delayed in mice vaccinated i.d. versus s.c. Naïve mice (Na) or mice immunized 7 weeks prior in the FP or ear with 10⁴ live *L. major* metacyclic promastigotes were rechallenged in the contralateral ear (ear-vaccinated mice) or both ears (naïve and FP-vaccinated mice) with 10³ metacyclic promastigotes. At various intervals following intradermal rechallenge the number of parasites in ears (A) and DLN (B) of immune and naïve C57BL/6 mice was assessed. Results shown represent parasite loads for individual ears or DLNs. Data are for six mice per group per time point.

and the naïve controls, while in this experiment the mice vaccinated in the FP already displayed significant immunity in the secondary challenge site ($P = 0.007$) (Fig. 3A). By 3 weeks postchallenge, the parasite loads in the FP-immunized mice were further reduced (geometric mean, 19), whereas in the ear-immunized mice the infections were still progressing (geometric mean, 4,096), though not as rapidly as in the naïve controls (geometric mean, 52,016). By 5 weeks, viable amastigotes had been completely eliminated from FP-vaccinated mice, whereas a reduction in the parasite burden in the ear-vaccinated mice was only just becoming apparent. The dramatic control of parasite growth by FP-vaccinated mice was again evident in the DLN (Fig. 3B), where the majority had no detectable dissemination and all were negative at 5 weeks postchallenge. In contrast, all of the ear-vaccinated mice

showed dissemination by 3 weeks, and mice still had parasites in their DLN at 5 weeks postchallenge.

The delayed clearance of parasites by ear-vaccinated mice compared to those vaccinated in the FP correlates with a delayed and weaker IFN- γ response at the site of secondary infection. In FP-vaccinated mice large numbers of IFN- γ -staining CD4⁺ lymphocytes quickly infiltrated the rechallenge site 1.5 weeks following challenge (Fig. 4). The frequency of IFN- γ -staining CD4⁺ cells peaked at 33% in the third week, concomitant with clearance of parasites, and then declined to about 14% at 5 weeks following complete elimination of parasites from the site. In contrast, IFN- γ -staining CD4⁺ cells were not found in significant amounts at the secondary site in ear-vaccinated mice until the third week of infection, where they accounted for only 10% of the CD4⁺ cells at both 3 and 5 weeks.

These data confirm that while vaccination with live organisms in the skin does confer immunity, the expression of immunity is delayed and less potent in comparison to that of the s.c. FP-vaccinated mice, and this delay allows parasites to expand in the inoculation site and to disseminate to the local draining node for up to 3 weeks postchallenge.

Analysis of the protective immunity expressed by s.c. versus i.d. vaccinated mice. The dramatic difference between the protective immunity expressed by the ear (i.d.)- versus FP (s.c.)-vaccinated animals prompted us to explore the possibility that IL-10-producing CD4⁺ CD25⁺ T_{REG} cells, known to regulate the persistence of parasites in the primary site of infection in the skin and to influence the expression of immunity to reinfection (6, 21), might be differentially activated in the vaccinated mice. Twenty weeks following vaccination with live parasites in the ear or FP, mice were rechallenged in the contralateral ear, for ear-vaccinated mice, or both ears, in FP-vaccinated mice. Two weeks following challenge, the secondary site was analyzed for parasite burden, numbers of IFN- γ - and IL-10-producing CD4⁺ T cells, and total cytokine production as assessed by ELISA. Both ear- and FP-vaccinated animals had significantly reduced parasite burdens compared to the naïve control, confirming the durable immunity (20 weeks) conferred by the use of live vaccine (Fig. 5A). Again, however, the protection in the FP-vaccinated mice was significantly more potent, with 100 times fewer parasites in the 2-week rechallenge site compared to the ear-vaccinated mice. Ear-vaccinated mice harbored both IFN- γ - and IL-10-producing CD4⁺ cells in the secondary site (Fig. 5B). There was, however, a greater number of CD4⁺ cells producing IL-10 compared to IFN- γ , representing 12.5% versus 8.4% of total CD4⁺ T cells, respectively. In comparison, FP-vaccinated mice harbored virtually no IL-10-producing cells (1.8% of all CD4⁺ cells) and considerably more IFN- γ -producing cells (16.3%). The decrease in total IFN- γ production by dermal cells in the rechallenge site in ear- versus FP-vaccinated mice was more variable when analyzed by ELISA, with some experiments showing comparable levels of total IFN- γ being secreted by cells in the rechallenge site following antigen restimulation in vitro (Fig. 5C). We attribute at least part of this IFN- γ secretion to non-CD4⁺ T cells, as indicated by the dot plot analyses shown in Fig. 5B. Interestingly, ELISA analysis of IL-10 production by dermal cells revealed increased levels of IL-10 in ear-vaccinated mice, and, more importantly, this IL-10 produc-

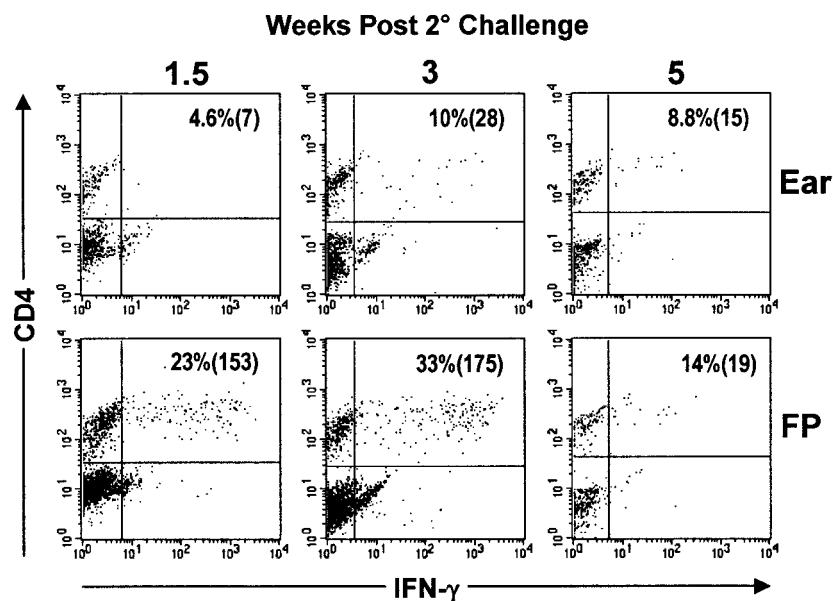


FIG. 4. Recruitment of IFN- γ -producing CD4⁺ T cells to a site of secondary challenge is delayed in mice vaccinated i.d. versus s.c. Mice immunized 7 weeks prior in the FP or ear with 10^4 live *L. major* metacyclic promastigotes were rechallenged in the contralateral ear or both ears with 10^3 metacyclic promastigotes. At various time points following intradermal rechallenge, cells from the ear dermis were restimulated in vitro with *L. major*-infected BMDDC and stained for surface markers and intracytoplasmic IFN- γ .

tion was antigen driven (Fig. 5D). The data suggest that antigen-specific IL-10 production by CD4⁺ T cells is more prevalent in the recall response of ear-immunized mice. Analysis of the phenotype of CD4⁺ cells at the secondary site revealed that 10% of CD4⁺ cells in FP-vaccinated mice also expressed the CD25 marker, versus 7% in ear-vaccinated mice and 10% in naïve mice (data not shown). These results indicate that, at a site of active infection, the CD25 marker is not necessarily a strong indicator of the presence of antigen-specific, IL-10 producing CD4⁺ T_{REG} cells.

The role of IL-10 and CD4⁺CD25⁺T_{REG} cells in the delayed expression of immunity in i.d.-vaccinated mice. Our finding that ear-vaccinated mice harbored increased numbers of IL-10-producing CD4⁺ cells that were rapidly recruited to the rechallenge site suggested that these cells might be playing a role in the suppression of immunity. To investigate whether IL-10 and/or CD25⁺ cells were modulating the immunity to reinfection in the ear-immunized mice, these animals were treated 10 weeks following primary infection with either anti-CD25, anti-IL-10R, or control antibodies and challenged with 10^3 metacyclics in the contralateral ear. Three weeks following rechallenge, the parasite load at the secondary site was determined. Treatment of ear-vaccinated mice with anti-CD25 antibodies significantly lowered the parasite burden at the secondary challenge site compared to control antibody treatment (Fig. 6), as previously reported (21). Treatment with anti-IL-10R antibody also significantly decreased the parasite load at the secondary site of infection. Interestingly, anti-CD25 and anti-IL-10R treatment did not convert ear-vaccinated mice to an optimally immune, FP-vaccinated phenotype, as parasite loads remained about 10-fold higher than those of FP-immunized mice, suggesting that other factors are also involved.

Comparison of the antigen load and immune response at the primary sites of infection. A possible explanation for the

difference in the efficacy of live vaccination s.c. in the footpad versus. i.d. in the ear dermis is that different antigenic loads are established during the acute or chronic phases of infection in the primary sites, resulting in the differential generation, expansion, and/or maintenance of *Leishmania*-specific memory cells. Two groups of mice were inoculated with 10^4 metacyclic promastigotes in the ear or FP, and the parasite loads at the injection site and DLN were determined at various time points following infection (Fig. 7). Three weeks following infection, the parasite loads were identical in the two injection sites as well as in the respective local lymph nodes draining these sites, suggesting that either site is equally suitable for parasite viability and growth during the acute phase of infection. At 7 weeks, during the peak of the adaptive immune response, parasite loads were higher at the site of infection and the DLN in FP- versus ear-vaccinated mice, although it is possible that the peak parasite burdens were comparable, established sometime between the 3- and 7-week time points chosen for analysis. At later time points, during the chronic phase of infection when the parasite load reaches a steady state (5), the DLNs of mice injected in the ear or FP showed comparable parasite numbers. In contrast, and unlike the result seen at 7 weeks, infected ears maintained a higher steady-state parasite load than that observed in footpads. The bias toward higher parasite loads at later time points in ear-vaccinated mice is reflected in the immune state maintained in their DLNs, which is biased slightly toward a “regulatory” phenotype, as shown by lower levels of IFN- γ and increased levels of IL-10 (Fig. 8). Levels of Ag-driven IL-4 and the frequency of IL-4-producing CD4⁺ T cells in both the primary site and the DLN of the primary site in ear- and FP-vaccinated mice were comparable and very low, <150 pg/ml and <1%, respectively, suggesting that the difference in the efficacy of vaccination is not controlled by a tissue-specific Th2 bias (data not shown).

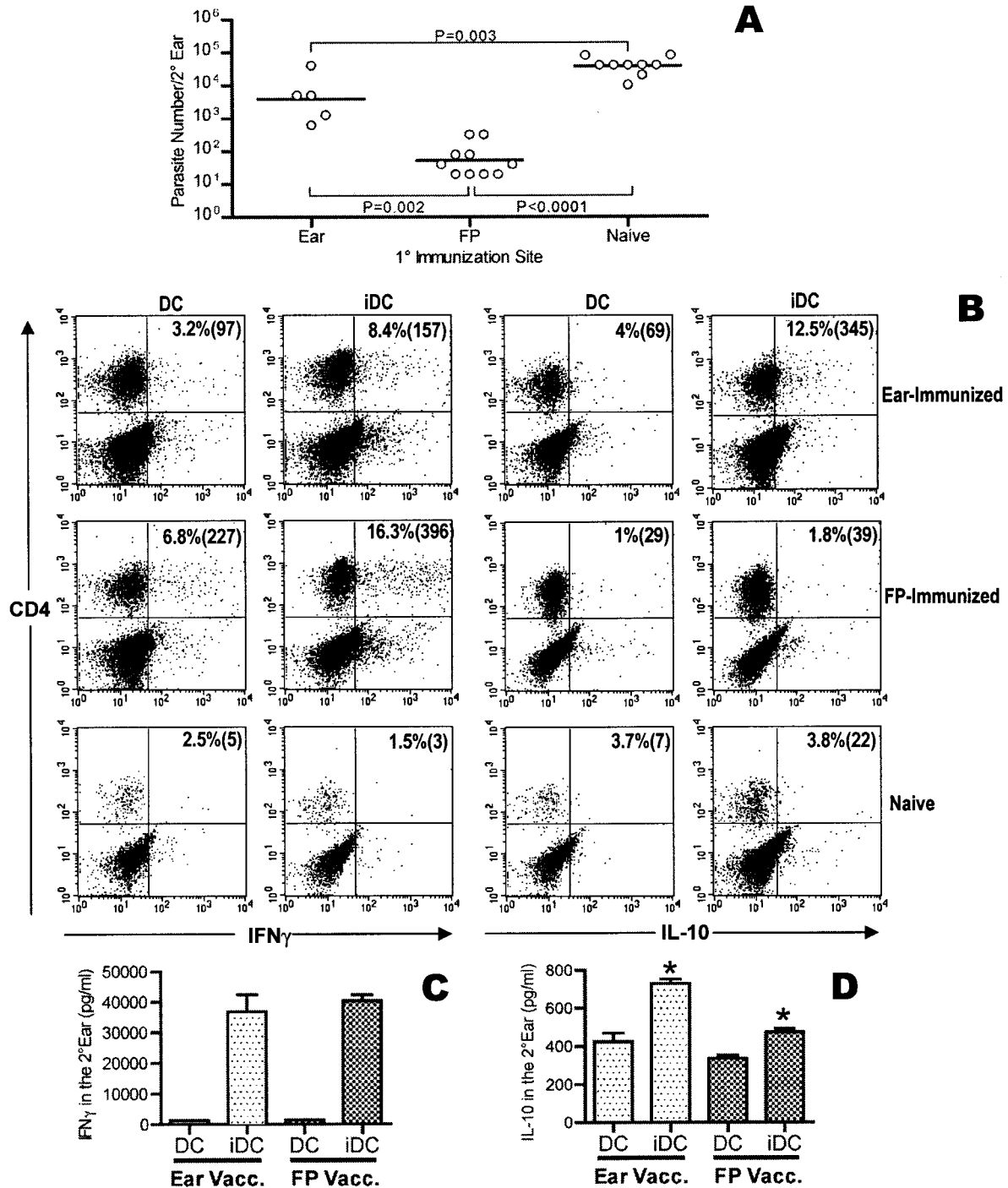


FIG. 5. Analysis of the rechallenge site in mice with chronic infection. Naïve mice (Na) or mice vaccinated 20 weeks prior with 10⁴ live *L. major* metacyclic promastigotes in the FP or ear were rechallenged in the contralateral ear or both ears with 10³ metacyclic promastigotes. Fourteen days following rechallenge, the secondary site was analyzed as follows: parasite load in the secondary ear dermis site (A); IFN- γ or IL-10 intracytoplasmic staining of CD4⁺ T cells isolated from the ear dermis following 24 h in vitro restimulation with BMDDC uninfected (DC) or infected with *L. major* (iDC) (B); IFN- γ production by dermal cells isolated from the secondary ear site measured by ELISA (C); and IL-10 production by dermal cells prepared as in panel C (D). Five mice per group were used. Results are representative of three repeat experiments. *, $P = 0.0001$ between iDC groups.

DISCUSSION

Vaccination of humans with live *L. major* parasites, resulting in a healed primary lesion, generates a highly protective im-

mune response, as demonstrated by a strong and long-lived resistance to disease upon reexposure (12, 16, 28). Despite the proven efficacy of live vaccines and the inability of other vaccination strategies to replicate the potency or durability of live

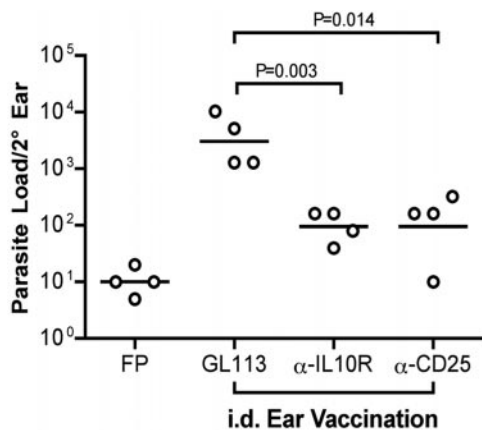


FIG. 6. Treatment with anti-IL-10R or anti-CD25 antibodies leads to reduced parasite loads at the secondary site of infection in i.d. ear-vaccinated mice. C57BL/6 mice, vaccinated in the FP or ear 10 weeks prior with 10^3 *L. major* metacyclic promastigotes were rechallenged in the contralateral ear with 10^4 *L. major* promastigotes. Ear-vaccinated mice were treated with 0.5 mg of anti-CD25, anti-IL-10R, or isotype control (GL113) antibodies i.p., 3 days prior to, on the day of, and 3 days following rechallenge. Parasite load in the secondary ear site was determined 3 weeks following rechallenge. Each data point represents the secondary challenge site obtained from one mouse. Results are representative of three repeat experiments.

L. major vaccines, there does not exist a careful analysis of the protective secondary response, including the early events associated with the control of infection in the secondary challenge site. Our analysis of the secondary immune response in mice vaccinated up to 4 to 5 months previously s.c. in the FP with live *L. major* parasites reveals a remarkably rapid immune response, characterized by the accumulation of *L. major*-specific IFN- γ -producing CD4⁺ T cells within 1.5 weeks following low-dose rechallenge in the ear dermis. The speed with which this response was able to completely clear viable parasites from the rechallenge site was surprising—as early as 2.5 weeks following reinfection—during which time there was little or no parasite dissemination into the DLN. Importantly, this level of protection conferred by vaccination with live parasites in a subcutaneous site was not achieved when mice were immunized with live *L. major* in the ear dermis, a compromise that we attribute, at least in part, to a greater frequency of IL-10-producing CD4⁺ CD25⁺ T cells that are generated by chronic infection in the skin and that home early to the rechallenge site.

Prior studies have suggested that the site of primary infection can influence the outcome of disease and the Th1/Th2 balance of the primary immune response (3, 9, 26, 27). In addition, Kirkpatrick et al. have shown that the site of primary infection involving different intradermal sites on the back can influence the severity of the primary lesion and the resolution of a secondary lesion following reexposure to *L. major* (18). These studies did not determine the nature of the immune response at the secondary site, and no mechanistic studies that might explain the different outcomes were carried out. In the current studies, a comparison of the early events associated with immunity in the rechallenge site revealed a stronger IFN- γ response and more rapid recruitment of IFN- γ -producing CD4⁺ cells in FP-vaccinated mice. In contrast, ear vacci-

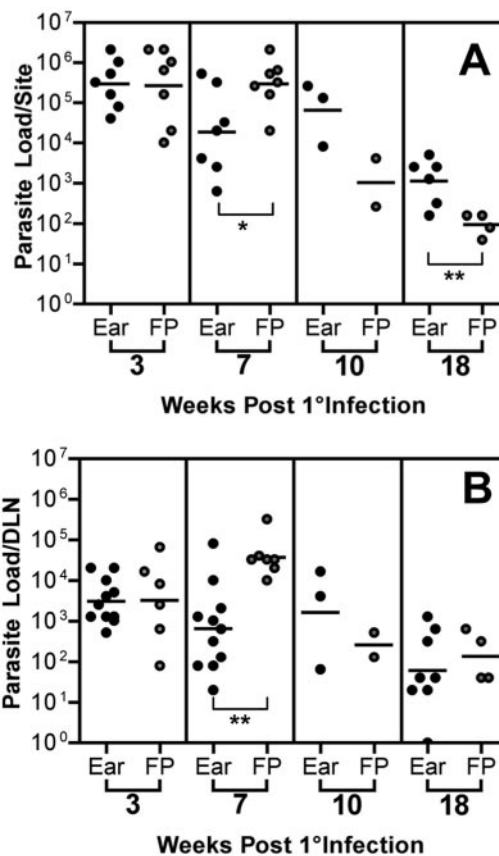


FIG. 7. Analysis of the parasite load during the course of primary infection in ear- and FP-immunized mice. The number of parasites at the site of infection (A) and DLN (B) in C57BL/6 mice was determined at various time points following immunization with 10^4 metacyclic *L. major* promastigotes in the ear or FP. Each data point represents an individual animal. *, $P < 0.05$; **, $P < 0.005$.

nation resulted in a slower recruitment of IFN- γ -producing CD4⁺ cells to the secondary challenge site, despite the fact that a relatively strong IFN- γ response was maintained in the primary DLN at the time of rechallenge. The defect appeared to be controlled not by a shift in the Th1/Th2 balance, as the chronic infection in either site generated low or undetectable levels of IL-4, but by a greater population of IL-10-producing CD4⁺ cells that were rapidly recruited to the secondary site. Analysis of total IL-10 production by lesion-derived cells using ELISA also demonstrated significantly higher levels of Ag-driven IL-10 production.

It was previously reported that CD25⁺ T_{REG} cells are the primary CD4⁺ T-cell source of IL-10 in the chronically infected ear dermis (6). It was also shown that adoptive transfer of lesion-derived CD25⁺ T_{REG} cells can compromise the expression of immunity in the rechallenge site and that anti-CD25 depleting antibody enhances parasite clearance (21). In the current studies we found that treatment of ear-vaccinated mice with either anti-CD25 or anti-IL-10R antibodies enhanced the early clearance of parasites from the secondary site. These observations suggest that in comparison to s.c. infection, chronic infection in the skin generates a greater population of IL-10-producing CD25⁺ T_{REG} cells that rapidly home to a

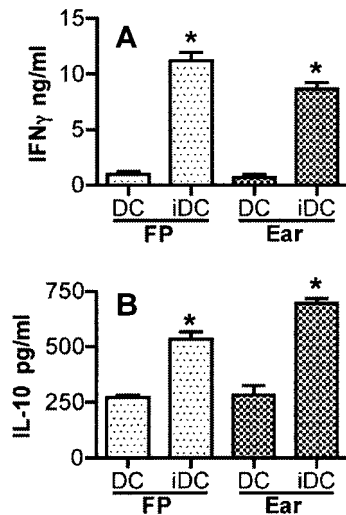


FIG. 8. Analysis of *L. major* Ag-specific cytokine production by cells derived from the DLN of the site of chronic, primary infection. Naïve C57BL/6 mice were infected in the FP or the ear 16 weeks prior with 10^4 *L. major* promastigotes. (A) IFN- γ production by DLN cells following in vitro restimulation with BMDDC infected (iDC) or uninfected (DC) with *L. major* as determined by ELISA. (B) IL-10 production by DLN cells as in panel A. *, $P < 0.001$ between iDC groups. The results are representative of two repeat experiments.

secondary site of infection, thus influencing immunity. The more “regulatory” phenotype observed following intradermal vaccination may be physiologically relevant. The skin and mucosa represent the most likely sites of exposure to foreign noninfectious antigens, infectious agents, and, perhaps most importantly, normal flora. Thus, these tissues would potentially benefit most from the presence of T_{REG} cells, as evidenced by the immunopathology associated with the absence of CD25⁺ T_{REG} cells in colitis, antigen-specific contact sensitivity, and dermatitis (2, 8, 19).

In our search for immune correlates of the powerful immunity elicited by vaccination with live organisms in a subcutaneous site, we found that the number of IFN- γ -producing CD4⁺ T cells diminished quickly after parasite clearance. Thus, the association of this response with vaccine potency may be underestimated in studies where the secondary immune response is assessed at late time points. Our studies also more critically evaluated vaccine potency by challenging up to 20 weeks post-vaccination, with little or no loss in the powerful protection conferred by vaccination with live parasites in a subcutaneous site.

Our observations in FP-vaccinated mice appear to reveal the ideal immune state, one in which a low-level, chronic infection at the primary site is established, presumably by a careful balance of T_{EFF} and T_{REG} cells that provides persisting antigen to maintain effector cells but does not compromise the full expression of concomitant immunity at a secondary site. In contrast, in ear-vaccinated mice, greater numbers of IL-10-producing T_{REG} cells are generated that maintain a higher level of persistent infection in the primary site and that are able to migrate into the secondary site of infection and suppress immunity. The consideration of this ideal immune state is important for the generation of an effective, safe *L. major*

vaccine and may also have implications for vaccination strategies against other intracellular pathogens.

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