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Hybrid Cell Vaccination Resolves *Leishmania donovani* Infection by Eliciting a Strong CD8$^+$ Cytotoxic T-Lymphocyte Response with Concomitant Suppression of Interleukin-10 (IL-10) but Not IL-4 or IL-13$^{\nabla}$‡

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There is an acute dearth of therapeutic interventions against visceral leishmaniasis that is required to restore an established defective cell-mediated immune response. Hence, formulation of effective immunotherapy requires the use of dominant antigen(s) targeted to elicit a specific antiparasitic cellular immune response. We implemented hybrid cell vaccination therapy in *Leishmania donovani*-infected BALB/c mice by electrofusing dominant *Leishmania* antigen kinetoplastid membrane protein 11 (KMP-11)-transfected bone marrow-derived macrophages from BALB/c mice with allogeneic bone marrow-derived dendritic cells from C57BL/6 mice. Hybrid cell vaccine (HCV) cleared the splenic and hepatic parasite burden, eliciting KMP-11-specific major histocompatibility complex class I-restricted CD8$^+$ cytotoxic T-lymphocyte (CTL) responses. Moreover, splenic lymphocytes of HCV-treated mice not only showed the enhancement of gamma interferon but also marked an elevated expression of the Th2 cytokines interleukin-4 (IL-4) and IL-13 at both transcriptional and translational levels. On the other hand, IL-10 production from splenic T cells was markedly suppressed as a result of HCV therapy. CD8$^+$ T-cell depletion completely abrogated HCV-mediated immunity and the anti-KMP-11 CTL response. Interestingly, CD8$^+$ T-cell depletion completely abrogated HCV-induced immunity, resulting in a marked increase of IL-10 but not of IL-4 and IL-13. The present study reports the first implementation of HCV immunotherapy in an infectious disease model, establishing strong antigen-specific CTL generation as a correlate of HCV-mediated antileishmanial immunity that is reversed by in vivo CD8$^+$ T-cell depletion of HCV-treated mice. Our findings might be extended to drug-nonresponsive visceral leishmaniasis patients, as well as against multiple infectious diseases with pathogen-specific immunodominant antigens.

Visceral leishmaniasis (VL), also known as kala-azar, caused by the intracellular parasite *Leishmania donovani*, is characterized by defective cell-mediated immunity (32). To date, no effective vaccines exist against VL, a disease that is fatal if left untreated and that has resulted in more than 100,000 deaths in recent epidemics in Sudan and India (26). Since any form of chemotherapeutic intervention potentially results in the development of drug-resistant forms (32, 47, 48), effective antileishmanial intervention should include immunologically defined antigen-based therapy targeted effectively to stimulate relevant effector and helper defense mechanisms. Moreover, failure to appreciate the mechanism and therapeutic targets governing protective immune response against *L. donovani* infection is posing difficulty in devising a suitable vaccine formulation.

Though not tested against any infectious diseases so far, hybrid cell vaccine (HCV) has yielded promising results in the treatment of human cancer. Hybrid cells prepared from the infusion of tumor and allogeneic dendritic cells induce a tumor-specific CD8$^+$ T-cell response resulting in disease regression or stabilization in melanoma, renal cancer, and breast cancer patients and causing only minor side effects (classified as grade I according to World Health Organization toxicity grading criteria) in a few patients, thus confirming the nontoxicity of the vaccine (3, 44, 50, 51). Hence, due to the feasibility of implementation and known ability of hybrid cell vaccination to generate vigorous major histocompatibility complex (MHC) class I-restricted CD8$^+$ cytotoxic T-lymphocyte (CTL) response, we used kinetoplastid membrane protein 11 (KMP-11) antigen-based HCV therapy in *L. donovani*-infected mice. Previously, we have established the efficacy of KMP-11 as an immunodominant candidate antigen for DNA vaccine against VL (6). Recently, we have dissected the remarkable antigenicity of KMP-11 by defining 29 MHC class I-restricted epitopes capable of inducing gamma interferon (IFN-γ) secretion by human CD8$^+$ T cells (5). In the context of the role of CD8$^+$ T cells in leishmaniasis, a dominant role of vaccine-induced CD8$^+$ T cells been implicated in cutaneous leishmaniasis (CL) since CD8$^+$ T-cell depletion resulted in the abrogation of the protective efficacy of the LACK-DNA vaccine (14). CD8$^+$ T

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cells were also acknowledged to be responsible for the establishment of resistance in the VL model (34, 43). However, in the CL model, activated $CD8^+$ T cells facilitated IFN-γ production and simultaneously masked the detrimental interleukin-4 (IL-4)-producing Th2 type of immune response (52). In contrast, our earlier observation suggested that the protective immune response against *L. donovani* infection by KMP-11 DNA vaccination associated with a strong antigen-specific CTL response and high IFN-γ generation surprisingly did not suppress an IL-4-producing Th2-type response (6). Due to the absence of a defined CTL marker in the hamster model, it was difficult to predict whether the CTL response that failed to dampen or suppress the Th2 response was restricted to the $CD8^+$ T-cell population (6). Moreover, it has been reported that, compared to untreated, active VL patients, the frequency of activated $CD8^+$ T cells substantially increased in asymptomatic and treated VL patients, along with high IFN-γ- and IL-4-producing $CD4^+$ T cells (15). Intriguingly, in the same study, both IFN-γ production and IL-4 production were found to be very low in active VL patients, a finding suggestive of a suppressive mechanism acting on both Th1 and Th2 subsets of T cells in the presence of a low frequency of activated $CD8^+$ T cells. In another clinical study, *Leishmania*-specific $CD8^+$ T cells have been identified as an important contributor to antileishmanial immunity in asymptomatic VL patients exhibiting a mixed Th1/Th2 response (25). *L. donovani*-reactive $CD4^+$ T cells clones secreting both IFN-γ and IL-4 have also been isolated from individuals who have recovered from VL after antimonial treatment (18). Thus far, no mechanism has been suggested to explain the existence of both IFN-γ-producing and IL-4-producing $CD4^+$ T-cell clones from chemotherapeutically cured or asymptomatic VL patients (15, 18, 25). Hence, due to the known ability of HCV to activate $CD8^+$ T cells, we reasoned that HCV therapy against *L. donovani* is likely to establish a correlation between $CD8^+$ T cells and the Th1/Th2 type of immune response. Increasingly, reports are suggesting that the relevant importance of the Th2 immune response is clearly *Leishmania* species dependent since a mixed Th1/Th2 protective response is associated with VL as opposed to the polarized Th1 protective response observed in CL (17, 18, 21, 27). Although IL-10 is universally acknowledged as a disease-promoting cytokines in both CL and VL, there are many conflicting reports of both IL-4 and IL-13 having opposite roles to play in VL and being associated with resistance (2, 6, 17, 28, 33, 36, 41, 42). Sacks and coworkers have previously demonstrated that IL-10 is regulated independently of IL-4 in patients with active VL and suggested that the source of IL-10 is not classically defined Th2 cells (16). Since addressing the cellular basis of coexistence of the mixed Th1/Th2 response in protective immunity in VL is essential for defining the successful implementation of a vaccine, our study focused on the HCV-induced production of the Th1 cytokine IFN-γ and the Th2 cytokines IL-4, IL-13, and IL-10. Our finding reveals that the HCV-induced $CD8^+$ T-cell-mediated antileishmanial protective immune response, despite augmenting IFN-γ production, specifically suppresses IL-10 but not IL-4 or IL-13 from splenic T cells, thus accounting for the mixed Th1/Th2 response observed in protection against *L. donovani* infection.

MATERIALS AND METHODS

Reagents and cell culture. HEPES, penicillin-streptomycin, $NaHCO_3$, 2-mercaptoethanol, brefeldin A, dimethyl sulfoxide, sodium azide, EDTA, ATP, and sucrose were purchased from Sigma Aldrich (St. Louis, MO). [^{3}H]thymidine was obtained from New England Nuclear (Boston, MA). The recombinant mouse cytokines rmIL-2, rmGM-CSF, rmIL-4, and rmTNF-α were purchased from BD Biosciences (San Diego, CA), and rmMCSF was purchased from Sigma. Cytokine enzyme-linked immunosorbent assay (ELISA) kits for IL-4, IL-10, and IFN-γ were obtained from BD Biosciences (San Diego, CA). An IL-13 ELISA kit was obtained from e-Bioscience (San Diego, CA). The $CD4^+$ magnetic activated cell sorting (MACS) antibody was purchased from Miltenyi Biotec. RAW 264.7 (*H-2*^{d}), a murine macrophage cell line, and 2.43, a murine lymphoblast secreting antibodies to Lyt2.2, were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 100 U of penicillin/ml, 100 μg of streptomycin/ml, 4 mM $NaHCO_3$, 20 mM HEPES, and 5×10^{-5} M 2-mercaptoethanol, along with 10% fetal calf serum (Invitrogen), at 37°C in the presence of a 5% CO_2 supply.

Animals, parasites, and infection. BALB/c (*H-2*^{d}) mice, 4 to 6 weeks old and reared in our institute facilities, and C57BL/6 (*H-2*^{b}) mice, 4 to 6 weeks old and purchased from NIN (Hyderabad, India), were used for experiments with the prior approval of the animal ethics committee of the Indian Institute of Chemical Biology (Kolkata, India). *L. donovani* strain AG83 (MHOM/IN/1983/AG83) was used for our experiments (6). AG83 was originally obtained from Indian kala-azar patients and was maintained in golden hamsters as described previously (6). Promastigotes obtained after transforming amastigotes from infected hamster spleen were maintained in M199 (Invitrogen) supplemented with antibiotics and 10% fetal calf serum at 22°C. For infection, 4- to 6-week-old BALB/c mice were injected with 10^6 promastigotes suspended in saline by the intracardiac route using a 28-gauge needle.

Generation of BM-Mϕ and BM-DC and transfection. Bone marrow-derived dendritic cells (BM-DC) were generated from bone marrow progenitors in the presence of rmGM-CSF and rmIL-4 (1). A total of 10^6 nonadherent bone marrow cells/ml, collected after passage of marrow from the tibias and femurs of BALB/c (*H-2*^{d}) or C57BL/6 (*H-2*^{b}) mice, were seeded in a 24-well plate in the presence of rmGM-CSF (150 U/ml) and rmIL-4 (75 U/ml) and then cultured for 3 days in a 37°C incubator with a 5% CO_2 supply. On day 3, nonadherent cells (2.5×10^6/2 ml/well) were again transferred and supplemented with complete medium and cytokines, and subsequently cultures were fed with rmGM-CSF and rmIL-4 on days 5 and 7. After 10 days, the nonadherent cells were collected. During the last 14 to 18 h of BM-DC culture, the cells were grown in the presence of rmTNF-α (20 ng/ml), providing DC maturation stimulus. Bone marrow-derived macrophages (BM-Mϕ) were obtained similarly from bone marrow cells of BALB/c (*H-2*^{d}) mice in the presence of 0.2 ng of rmGM-CSF/ml and 50 ng of rmM-CSF/ml, followed by incubation at 37°C with a 5% CO_2 supply for 24 h. Nonadherent cells were transferred to a new well with the same medium and cytokines and then cultured for an additional 5 to 7 days at 37°C in a CO_2 incubator (23).

Endotoxin-free plasmids were isolated by using an EndoFree Plasmid Mega kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions and used for the transfection of BM-DC and BM-Mϕ with Effectene reagent (QIAGEN) according to the manufacturer's protocols. The transfection efficiency of BM-Mϕ and BM-DC was estimated by counting red fluorescent cells under an inverted fluorescence microscope and by flow cytometry after transfection with a red fluorescent protein carrying plasmid pDsRED (BD Clontech). BM-DC showed a transfection efficiency of <6%, while BM-Mϕ showed a transfection efficiency of >18% (9).

Electrofusion parameters for hybrid cell generation and determination of fusion efficiency. Electrofusion of allogeneic BM-DC with KMP-11 DNA-transfected syngeneic BM-Mϕ and syngeneic BM-DC was performed as described previously (37) with several modifications. Briefly, partnering cells were washed thoroughly with sterile 0.3 M isotonic sucrose solution. Immediately before fusion, 2×10^7 of viable untransfected allogeneic BM-DC/ml and 2×10^7/ml of viable syngeneic BM-Mϕ or BM-DC transfected with KMP-11 DNA/ml were mixed at a ratio of 1:1 to yield a density of 2×10^7/ml in an inert wax-coated electroplated cuvette (Bio-Rad, Hercules, CA). After a series of standardization events for electrofusion, partnering cells were subjected to a range of alignment voltage (10 to 40 direct current V) and pulse voltage (100 to 400 V) (see Fig. S1 in the supplemental material). For therapeutic vaccination purposes, hybrids of KMP-11-transfected BM-Mϕ and BM-DC were used for their high percentage of fusion (>30%), which corresponded to a high viability (>90%) and greater transfection efficiency of macrophages. Maximum fusion and viability were ob-

served at an alignment voltage of 20 V (50 V/cm) with simultaneous application of 625 V/cm of square wave pulse (see Fig. S1 in the supplemental material). Fusion of KMP-11-transfected BM-DC with allogeneic BM-DC was not considered for HCV since DC-DC hybrid formation never exceeded 15.5%, with a corresponding low viability (<43%) at the tested ranges of alignment and pulse voltage. For analysis of a true fusion event, hybrid cells were analyzed by flow cytometry and further verified by fluorescence microscopy. Briefly, prior to electrofusion allogeneic BM-DC were labeled with intracellular dye CFSE (Molecular Probes, Inc., Eugene, OR), and syngeneic BM-Mϕ after KMP-11 transfection were stained with the membrane dye PKH26 (Sigma) according to the manufacturer's instructions and subsequently subjected to electrofusion procedure. The fusion efficiencies were >30% as evident from double-stained cells detected by flow cytometry (Becton Dickinson, San Jose, CA) and as further verified by fluorescence microscopy (Leica Microsystems, GmbH, Wetzlar, Germany) when superimposed (see Fig. S1 in the supplemental material). Expression of CD11b and CD11c was also determined to ascertain the purity of BM-Mϕ and BM-DC by flow cytometry, along with the expression of MHC class I, MHC class II, CD80, and CD86 of the partnering cells that showed high expression of all of these cell surface molecules (data not shown). Hence, for HCV preparation, partnering cells suspended in isotonic sucrose solution were first dielectrophoretically aligned to establish close cell-cell contact by applying a low voltage (20 V direct current for 20 s) in a spatially nonuniform electric field in an electrofusion chamber, followed by a simultaneously applied single square pulse of 625 V/cm (Gene Pulser Xcell; Bio-Rad) (55). After electrofusion, the cuvette was left undisturbed for 5 min before the cells were suspended in relaxation buffer (100 mM KCl, 3 mM NaCl, 1.25 mM EDTA, 10 mM HEPES, 0.5 mM ATP) for 15 min for complete membrane overlap. Cell viability, determined each time after electrofusion by dye exclusion, was usually >90%.

Therapeutic vaccination and in vivo CD8$^+$ T-cell depletion protocol. For vaccination, BALB/c mice infected with *L. donovani* for 2 months were divided into several groups receiving one to six intravenous (i.v.) injections of 5×10^5 cells/animal/100 μl of phosphate-buffered saline given 5 days apart. The following types of cells were used for therapeutic vaccination purposes: (i) syngeneic BM-DC transfected with KMP-11 DNA (syngeneic BM-DC–KMP-11); (ii) syngeneic BM-Mϕ transfected with KMP-11 DNA (syngeneic BM-Mϕ–KMP-11): (iii) untransfected syngeneic BM-DC (syngeneic BM-DC); (iv) untransfected syngeneic BM-Mϕ (syngeneic BM-Mϕ); (v) hybrids of KMP-11 DNA-transfected syngeneic BM-Mϕ and allogeneic BM-DC (HCV); and (vi) hybrids of untransfected syngeneic BM-Mϕ and allogeneic BM-DC (syngeneic BM-Mϕ+allogeneic BM-DC). Therapeutic vaccination with blank vector (pCMV-LIC)-transfected syngeneic BM-DC/BM-Mϕ and hybrids of blank vector-transfected syngeneic BM-Mϕ and allogeneic BM-DC was also performed. For a dose kinetics study of mixed (unfused) cells and pulsed, electrofused (hybrid cells) cells, mice were sacrificed after 15 days of receiving last injection(s) (one to six doses) of mixed unfused cells or hybrid cells (HCV). For organ parasite burden detection and all other experiments, mice were sacrificed 15 days after receiving six doses of HCV or six injection doses of different cell types as described above. For CD8$^+$ T-cell depletion, groups of mice therapeutically vaccinated with HCV were treated intraperitoneally with 1 mg of anti-CD8 (2.43) antibody 24 h before every HCV therapy.

Determination of splenic and hepatic parasite burden by serial dilution method. The parasite burden was quantified in spleen and liver tissue by serial dilution assay as described previously (6). Wells were examined for viable and motile promastigotes every 3 days, and the reciprocal of the highest dilution that was positive for parasites was considered to be the parasite concentration per milligram of tissue.

Parasite soluble antigen preparation and rKMP-11 protein purification. The soluble leishmanial antigen (SLA) used in the T-cell proliferation assay and the CTL assay was prepared from stationary-phase promastigotes of *L. donovani* (AG83) according to a published protocol (6). rKMP-11 protein under native conditions was purified by Ni-nitrilotriacetic acid affinity column from pQE-30 plasmid (kindly provided by H. Moll, University of Wurzburg, Wurzburg, Germany) containing KMP-11 open reading frame aligned in frame with the His tag as described previously (6).

T-cell proliferation and CTL assay. A T-cell proliferation assay was performed as described previously (6). Briefly, a single cell suspension (10^5 cells/well) of splenocytes from different experimental groups of mice as described above was plated in triplicate in 96-well plates and allowed to proliferate for 3 days at 37°C in a 5% CO_2 incubator in the presence of different SLA concentrations (0.5, 5, or 50 μg/ml). Eighteen hours before they were harvested, the cells were pulsed with 1 μCi (6.7 Ci/mmol) of [^{3}H]thymidine/well. [^{3}H]thymidine uptake, as an index of proliferation, was measured by a liquid scintillation counter (TRI CARB 2100TR; Packard).

For the CTL assay, nonadherent splenocytes from HCV-treated mice (with or without depletion of CD8$^+$ T cells) and other different experimental groups of mice, as mentioned above, were expanded with SLA for 7 days. Recombinant IL-2 (20 U/ml) was added 3 days later (41). After this, effector T cells were incubated with ^{51}Cr-labeled targets (RAW 264.7 cells transfected either with pCMV-LIC, pCMV-LIC KMP-11, or pEGFP-N1) in round-bottom 96-well plates (200 μl) at different effector/target (E:T) ratios (6:1, 12.5:1, 25:1, or 50:1) for 4 h. Target cells (10^6) were labeled with 100 μCi of Na_2CrO_4 (2.5 mCi/38.4 μg/ml; BARC, Mumbai, India) as described previously (6). After 4 h of incubation,100 μl of culture supernatant after centrifugation was collected and counted in triplicates in a liquid scintillation counter (TRI CARB 2100TR; Packard). Specific lysis was calculated according to the following formula: % specific lysis = [(sample − spontaneous release)/(maximum release − spontaneous release)] × 100.

RNA isolation and quantitative transcript analysis of IFN-γ, IL-4, IL-13, and IL-10 from splenic CD4$^+$ T lymphocytes stimulated with rKMP-11. Splenic lymphocytes (5×10^6/ml) were stimulated with rKMP-11 protein (5 μg/ml) for 18 h. Total RNA from MACS-purified CD4$^+$ T cells from *L. donovani*-infected and HCV-treated (with or without depletion of CD8$^+$ T cells) BALB/c mice were isolated according to the RNeasy minikit isolation procedure (QIAGEN) and was individually analyzed (eight animals/group) by real-time reverse transcription-PCR. Briefly, 2-μg samples of RNA from different experimental groups of mice were first utilized for cDNA synthesis by random hexamers (Invitrogen) using Superscript II (Invitrogen) and PCR was performed as described previously (12). Briefly, a common master mix (SYBR green JumpStart Taq ReadyMix; Sigma), gene-specific primers, and cDNA were used for amplification in an iCycler PCR detector (Bio-Rad) according to the manufacturer's instructions. The cycling conditions for all of the genes of interest were 12 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at a gene-specific temperature for 30 s, and extension at 72°C for 45 s. The expression of a target gene was quantified by using the formula $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = [C_{T(\text{target})} - C_{T(\beta\text{-actin})}]_{\text{treated}} - [C_{T(\text{target})} - C_{T(\beta\text{-actin})}]_{\text{control}}$. Each gene was normalized to the housekeeping gene (β-actin) before the fold change was calculated to account for variations between different samples. After each real-time reaction, a melting curve was generated to ensure that only gene-specific PCR product was generated.

The following primers were used to amplify the following genes as described by Grogan et al. (12) except β-actin primers: IL-10, sense (5′-GGTTGCCAAG CCTTATCGGA-3′); IL-10, antisense (5′- ACCTGCTCCACTGCCTTGCT-3′); IL-4, sense (5′-AGATCATCGGCATTTTGAACG-3′); IL-4, antisense (5′-TTT GGCACATCCATCTCCG-3′); IFN-γ, sense (5′-GGATGCATTCATGAGTAT TGC-3′); IFN-γ, antisense (5′-CCTTTTCCGCTTCCTGAGG-3′); IL-13, sense (5′-GCTTATTGAGGAGCTGAGCAACA-3′); IL-13, antisense (5′-GGCCAG GTCCACACTCCATA-3′); β-actin, sense (5′-AGAGGGAAATCGTGCGTGA C-3′); and β-actin, antisense (5′-CAATAGTGATGACCTGGCCGT-3′).

Analysis of quantitative expression of the T-cell differentiation transcription factors T-bet and GATA-3. To study the expression of T-bet and GATA-3, splenocytes (5×10^6/ml) from different groups of infected and HCV-treated mice were stimulated with 4×10^6 live promastigotes/ml for 6 days (20). Afterward, CD4$^+$ T cells from promastigote-stimulated splenocytes, after Ficoll gradient, were purified by MACS and used for RNA isolation. cDNA was synthesized from 2 μg of RNA according to the above-described conditions, and quantitative PCR was performed under the following cycling conditions: 95°C for 12 min, followed by 40 cycles of 95°C for 30 s, 60°C for 60 s, and 72°C for 45 s. The following primers were used to amplify the indicated genes as described by Grogan et al. (12): GATA-3, sense (5′-AGAACCGGCCCCTTATCAA-3′); GATA-3, antisense (5′-AGTTCGCGCAGGATGTCC-3′); T-bet, sense (5′-CA ACAACCCCTTTGCCAAAG-3′); and T-bet, antisense (5′-TCCCCCAAGCA GTTGACAGT-3′).

Measurement of IFN-γ, IL-4, IL-13, and IL-10 protein levels in rKMP-11 stimulated macrophage-depleted splenic lymphocyte culture supernatant. Single cell suspensions (5×10^6/ml) of pooled splenocytes (four mice/group), depleted of macrophages by the application of two successive 90-min plastic plate adherence procedures as described previously (11), from different experimental groups of mice were stimulated with 5 μg of rKMP-11 protein/ml for 72 h in a 37°C incubator with a 5% CO_2 supply. All of the supernatants were collected and frozen at −70°C for subsequent analysis. IFN-γ, IL-4, IL-10, and IL-13 were evaluated from culture supernatants by cytokine ELISAs according to the manufacturer's protocol. The detection limits for the cytokines were as follows: IFN-γ, 31.3 pg/ml; IL-4, 7.8 pg/ml; IL-13, 30 pg/ml; and IL-10, 31.3 pg/ml.

Statistical analysis. A paired two-tailed Student *t* test was used for statistical analysis of the data. *P* values of <0.05 were considered statistically significant.

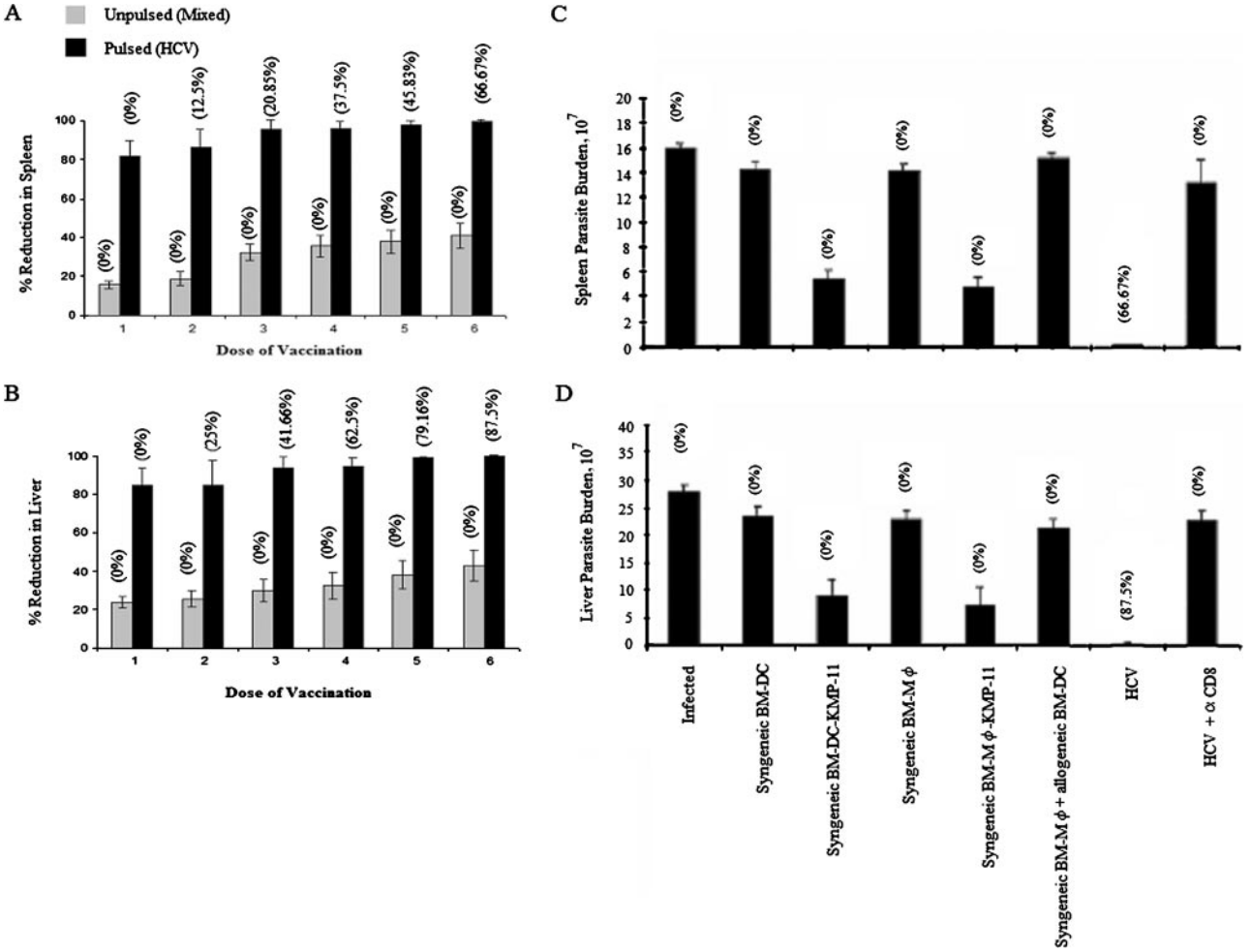

FIG. 1. HCV therapy in *L. donovani*-infected mice confers sterile protection that is completely abrogated by $CD8^+$ T-cell depletion. For vaccination, *L. donovani*-infected BALB/c mice were treated 60 days postinfection with i.v. injections of any one type of the cells mentioned in Materials and Methods. Therapeutically treated mice were sacrificed 15 days after the last injection. The data represent the means ± the SE for 24 animals per group. Figures in parentheses above the bars indicate percentages of mice showing complete absence of parasites in the spleen or liver as determined by serial dilution assay. (A and B) Comparative dose-response results for mixed cells and electrofused cells (HCV) in *L. donovani* (AG83)-infected BALB/c mice (2 months postinfection) receiving one to six i.v. injections of unpulsed (mixed) cells or pulsed (hybrid cells) in terms of reduction of parasite burden (bar diagram) and sterile cure (data in parentheses) in the spleen (A) and liver (B). In infected animals, the splenic parasite burden ranged from $4 \times 10^{-7} \pm 0.42 \times 10^{-7}$ (at the time of assessing the result of one HCV dose) to $15.4 \times 10^{-7} \pm 0.34 \times 10^{-7}$ (at the time of assessing the result of six doses of HCV). Similarly, the hepatic parasite burden in infected mice ranged from $7.6 \times 10^{-7} \pm 1.06 \times 10^{-7}$ (at the time of assessing the result of one HCV dose) to $27.7 \times 10^{-7} \pm 1.46 \times 10^{-7}$ (at the time of assessing the result of 6 doses of HCV). (C and D) Organ parasite burden of infected BALB/c mice receiving six i.v. injections of either HCV (with or without depletion of $CD8^+$ T cells) or other indicated cell types at 5-day interval. At 24 h before each HCV therapy, the mice were treated with 1 mg of anti-CD8 (2.43) antibody for successful depletion of $CD8^+$ T cells. Splenic (C) and hepatic (D) parasite burdens of infected BALB/c mice therapeutically treated with the above-mentioned cell types. The percentages of mice showing sterile cure are indicated in parentheses above the bars of the respective groups.

The results are represented as means ± the standard error (SE) as indicated in the figure legends.

RESULTS

Therapeutic vaccination with HCV clears both the splenic and the hepatic parasite burden that is completely abrogated by in vivo $CD8^+$ T-cell depletion. The virulent *L. donovani* strain AG83 (MHOM/IN/1983/AG83) used in the present study kinetics of infection different from that of the Sudanese (LV9 and LV82) and Ethiopian strains of *L. donovani* used in other studies (28, 40). After AG83 challenge, an exponential rise in both the splenic and the hepatic parasite burdens occurs until 5 months postinfection in a BALB/c mice model (29, 4). Since a cure from late-stage infection, at which the reversal of immune dysregulation is most critical, is the most desirable indicator of immunotherapeutic efficacy, we tried HCV therapy in a BALB/c mouse model at 60 days postinfection. We initially studied the detailed dose kinetics of mixed (unpulsed) and hybrid cells and its efficacy in parasite reduction from both the of the affected organs: spleen and liver (Fig. 1A and B). Considering the percentage of hybrid cell formation (>30%) and transfection efficiency of BM-Mϕ (>18%), a single dose of

HCV effectively introduced $>3 \times 10^4$ KMP-11-transfected fused cells. The dose response of HCV showed its remarkable therapeutic potential since a >80% reduction of splenic and hepatic parasite load was observed in animals treated with a single dose of HCV. More significantly, two doses of HCV resulted in completely undetectable amastigotes in 3 of 24 and 6 of 24 mice in the spleen and the liver, respectively. Comparative evaluation of therapeutic vaccination efficacy achieved by mixed, unpulsed cells (KMP-11-transfected syngeneic BM-Mϕ mixed with allogeneic BM-DC) showed a maximum of 25% reduction in organ parasite burden after two doses, reaching a maximum reduction of ~45% at six vaccination doses (Fig. 1A and B). This result established hybrid cell formation as an essential requirement for vaccine efficacy, whereas at a maximum of six doses of HCV the spleens of >66% of mice (16 of 24 mice) treated with HCV showed a complete absence of promastigotes in the serially diluted culture. The remaining 33.33% of HCV-treated mice (8 of 24 mice) showed a >98% reduction in splenic parasite burden, as determined by serial dilution assay (Fig. 1C). At a similar dose, complete clearance of hepatic parasite burden was observed in 87.5% of the hybrid cell vaccinated mice (21 of 24 mice), while the remaining animals (3 of 24 mice) showed ~99% reduction in the liver parasite burden, as determined by standard serial dilution assay (Fig. 1D). Although less sensitive than the serial dilution assay, the gold standard for detecting amastigotes for determining a clinical cure, microscopic evaluation of the parasite burden in Giemsa-stained smears was also attempted in our study (45, 46, 53). If no parasites are identified by this procedure in the splenic aspirate, the patients are said to be "cured" (45). Remarkably, no amastigotes could be detected by light microscopy (data not shown) in impressions of Giemsa-stained tissue stamp smears of transverse sections of spleens and livers from all of the HCV-treated mice that could be described as the classical definition of a "cure." To evaluate the contribution of an allo-MHC response with a syngeneic MHC response, the therapeutic efficacy of HCV was compared to KMP-11 DNA-transfected syngeneic antigen-presenting cells (APC). Hence, KMP-11-transfected syngeneic BM-DC or KMP-11-transfected syngeneic BM-Mϕ were also injected six times i.v. every 5 days. Comparative doses of KMP-11-transfected syngeneic BM-DC–BM-Mϕ could only confer ~ 64% and ~70% clearance of the splenic parasite burden ($P < 0.0005$) and ~67% and ~74% reduction of the hepatic parasite burden ($P < 0.0005$), respectively (Fig. 1C and D). Unlike HCV-treated mice, a sterile cure was not observed in any of transfected APC-treated mice. Neither the control groups of untransfected syngeneic BM-Mϕ or BM-DC ($P < 0.5$) nor the hybrids of untransfected syngeneic BM-Mϕ and allogeneic-BM-DC ($P > 0.05$) could induce any significant protection in organ parasite burden. Expectedly, therapeutic vaccination with blank vector-transfected syngeneic BM-DC, blank vector-transfected syngeneic BM-Mϕ, and blank vector-transfected fused cells could not confer any protection (data not shown).

Since the main objective of HCV therapy relies on its potential to generate a vigorous $CD8^+$ CTL response, we tried to assess the role of $CD8^+$ T cells in HCV immunotherapy by depleting $CD8^+$ T cells. In these experiments, depletion of $CD8^+$ T cells was preferred at the time of vaccination rather than at the time of infection to limit the induction of HCV-mediated antigen-specific $CD8^+$ T cells but not to limit the endogenous repertoire of $CD8^+$ T cells during the natural course of infection. It was observed that $CD8^+$ T-cell depletion completely abrogated HCV efficacy where the $CD8^+$ T-cell-depleted mice showed a high amastigote burden ($13.2 \times 10^{-7} \pm 1.88 \times 10^{-7}$ in the spleen and $22.5 \times 10^{-7} \pm 1.95 \times 10^{-7}$ in the liver). Similar to the observation of Gurunathan et al. (14), we observed that the administration of anti-CD8 (2.43) did not alter the frequency and function (in terms of IL-12 production) of the $CD11c^+$ $CD8^+$ DC population (data not shown), known to be involved in CTL priming (7), whereas >98% of the $CD3^+$ $CD8^+$ T cells were successfully depleted. Thus, it was conclusively established that $CD8^+$ T cells play a cardinal role in the HCV-mediated curative response.

HCV induces a strong anti-KMP-11 $CD8^+$ CTL response in *L. donovani*-infected BALB/c mice that is abolished by CD8 depletion. Abrogation of protective efficacy of LACK-DNA in the murine CL model due to $CD8^+$ T-cell depletion is suggestive of vaccination-induced dominant role of the $CD8^+$ T-cell-mediated protective immune response, although the role of $CD8^+$ restricted CTLs has not been exclusively implicated in these studies (14). Thus, we proceeded to measure KMP-11-specific CTL generation in therapeutically cured HCV-treated mice. Splenocytes stimulated for 7 days with SLA from HCV-treated mice showed a 58% lysis of ^{51}Cr-labeled KMP-11-transfected syngeneic Mϕ (RAW 264.7) at the highest E:T ratio (50:1; $P < 0.0005$). KMP-11-transfected BM-DC or KMP-11-transfected BM-Mϕ treated mice showed maximal lysis values of 29 and 31%, respectively, at an E:T ratio of 50:1 ($P < 0.005$) (Fig. 2). The infected and other control groups of mice showed <9% lysis of radiolabeled targets at a 50:1 E:T ratio and did not differ significantly between the infected group and hybrids of the untransfected syngeneic BM-Mϕ and allogeneic BM-DC treated control groups, a finding suggestive of a severely impaired $CD8^+$ CTL response due to *L. donovani* infection in the BALB/c model. Specificity of lysis was further corroborated by the failure of the nonadherent splenic effector T cells to lyse syngeneic Mϕ transfected with either enhanced green fluorescent protein expressing pEGFP-N1 construct or transfected with blank vector pCMV-LIC (data not shown). The role of allogeneic MHC in generating a strong CTL response was also supported by the observation that mice treated with HCV showed a statistically significant level of CTL generation (18% lysis) even at the lowest E:T ratio (6:1; $P < 0.05$), whereas KMP-11-transfected syngeneic BM-Mϕ or BM-DC treated mice failed to show significant lysis at the lowest E:T ratio. The CTL response was completely impeded in $CD8^+$ T-cell-depleted mice, resulting in the failure of lysis of KMP-11-transfected targets, confirming an essential role of $CD8^+$ CTL in the HCV-mediated curative response.

SLA-induced T-cell proliferation in HCV-treated mice is impaired by CD8 depletion. The cell-mediated immune response is impaired in VL patients, as well as in experimental models, and is marked by T-cell anergy specific to *Leishmania* antigens (6). Since hybrid cell vaccination could reverse impaired cell-mediated immune response by inducing strong CTL generation, we were interested in determining the effect of HCV therapy in an SLA-specific T-cell proliferative response. All of the partially protected and HCV-treated mice showed >4-fold enhancement ($P < 0.0005$) of the proliferative

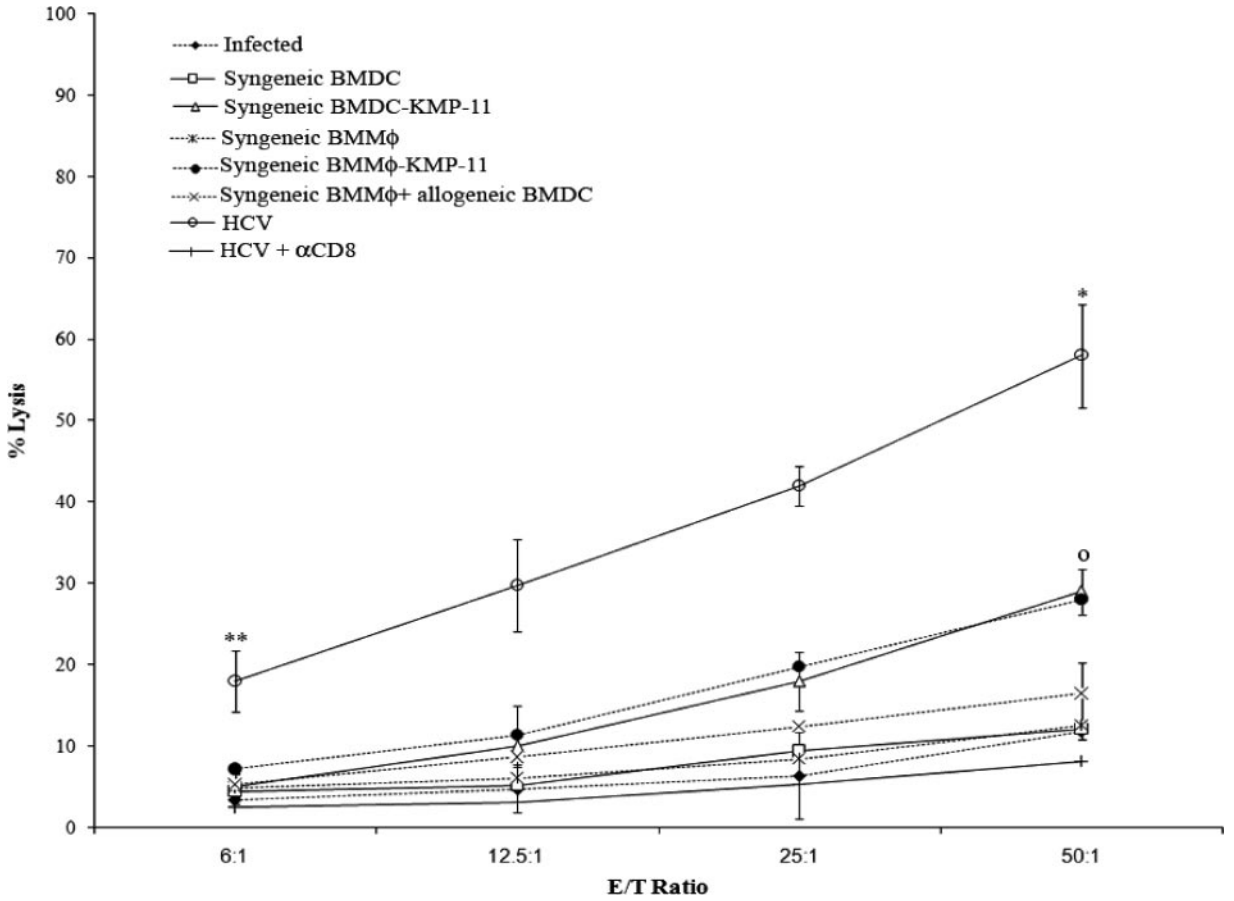

FIG. 2. HCV elicits a vigorous anti-KMP-11-specific CTL response in *L. donovani*-infected mice that is completely abrogated by $CD8^+$ T-cell depletion. CTL generation was assayed by a standard ^{51}Cr release assay in mice receiving six doses of HCV (with or without depletion of $CD8^+$ T cells) with the other indicated groups. Spontaneous release was <5%. The results are representative of five individual experiments (five animals/group/experiment), and the data represent the mean ± the SE. At both 50:1 and 6:1 E:T ratios, hybrid cell-vaccinated mice showed significant lysis (*, $P < 0.0005$; and **, $P < 0.0001$, respectively) with respect to infected controls, while syngeneic KMP-11-transfected APC-vaccinated mice showed statistically significant lysis of targets only at 50:1 (**o**, $P < 0.005$) compared to infected controls. Anti-CD8 Abs treated hybrid cell vaccinated mice were unable to generate any effective CTL response as reflected in the figure.

response versus the infected control at an SLA stimulation of 5 μg/ml, and HCV-treated mice showed a ~2-fold greater proliferation index ($P < 0.05$) compared to KMP-11-transfected syngeneic BM-Mϕ or KMP-11-transfected syngeneic BM-DC treated mice at a 5-μg/ml SLA stimulation (Fig. 3). $CD8^+$ T-cell-depleted HCV-treated mice showed similarly impaired T-cell proliferation compared to infected controls ($P <$ 0.5).

Only IL-10, and not IL-4 or IL-13, is considerably downregulated in HCV-treated mice at both mRNA and protein levels, which is reversed after $CD8^+$ T-cell depletion. In clinical scenarios, an increase in both IFN-γ- and IL-4-producing $CD4^+$ T cells has been observed in VL patients that have recently recovered (15). Moreover, $CD4^+$ T cells isolated from asymptomatic and chemotherapeutically cured VL individuals exhibited both Th1 and Th2 cytokine profiles, such as IFN-γ and IL-4, respectively (25). Thus, we initially proceeded to measure the transcriptional expression of the Th1 signature cytokine IFN-γ and the Th2 cytokines IL-4, IL-13, and IL-10 from rKMP-11-stimulated MACS-purified splenic $CD4^+$ T cells of HCV-treated (with or without depletion of $CD8^+$ T cells) and infected mice by real-time PCR (Fig. 4A). After HCV-therapy, IFN-γ transcripts in $CD4^+$ T cells were found to be expressed 3.65-fold more compared to infected controls (Fig.4Aa). Among Th2 cytokines, IL-4 mRNA was enhanced 8.8-fold, whereas IL-13 was enhanced 4.43-fold in HCV-treated mice compared to infected controls (Fig. 4Ac and d). On the other hand, IL-10 mRNA was downregulated by 24.68-fold after HCV therapy (Fig.4Ab). Due to $CD8^+$ T-cell depletion, IFN-γ was downregulated by 2.44-fold, IL-4 was downregulated by 5.51-fold, and IL-13 was downregulated by 3.54-fold with respect to the infected control. Surprisingly, IL-10 mRNA in splenic $CD4^+$ T cells was significantly upregulated by 2.12-fold versus the infected control due to $CD8^+$ T-cell depletion ($P < 0.005$). In fact, the IL-10 transcript was increased by >50-fold by CD8 depletion of HCV-treated mice compared to treated mice with an intact $CD8^+$ T-cell compartment. The results suggest that IL-10 mRNA is the only Th2 cytokine that was strongly downregulated, while other two Th2 cytokines—IL-4 and IL-13—were moderately upregulated, along with IFN-γ, after HCV therapy. Thus, the effect of depletion on IFN-γ, IL-4, and IL-13 from $CD4^+$ T cells was

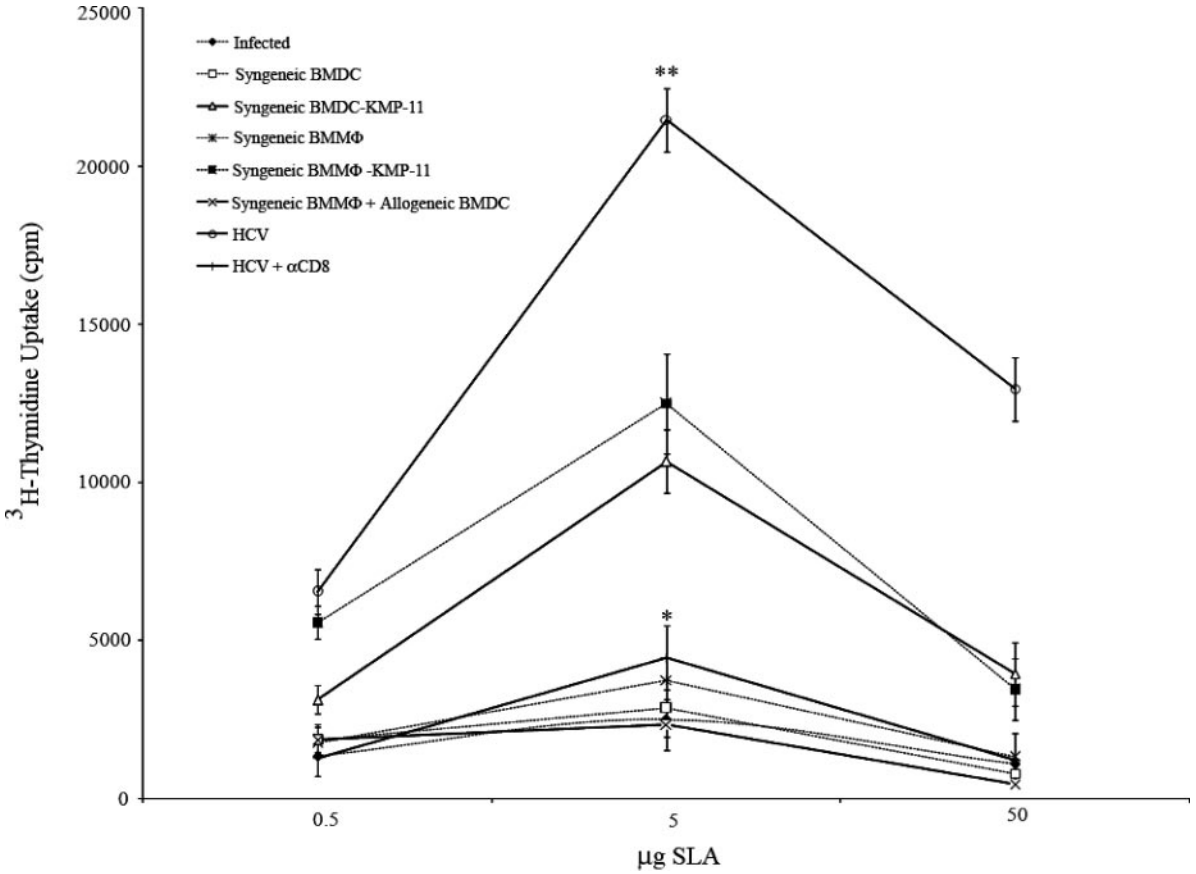

FIG. 3. HCV-treated mice restore T-cell anergy to SLA that is significantly impaired by CD8 depletion. The results are representative of three individual experiments (five animals/group), and the data represent the means of triplicate wells ± the SE. Proliferation was measured by determining the [^{3}H]thymidine incorporation. At a 5-μg/ml SLA stimulation, the double asterisks represent $P < 0.0001$ for HCV and the single asterisk represents $P = 0.1219$ for HCV-treated CD8$^+$ T-cell-depleted mice with respect to the infected group.

similar since all were significantly downregulated ($P < 0.05$). These observations suggest that activated CD8$^+$ T cells induced by HCV therapy override the suppressive effect of IL-10 by elevating both Th1 cytokine IFN-γ and Th2 cytokines IL-4 and IL-13.

Based on these observations, we reasoned that the suppressive effect of IL-10 might act on both Th1 and Th2 differentiation of CD4$^+$ T cells, thereby impeding activation of both effector T helper subsets. Hence, we wanted to study the mRNA expression profiles of the CD4$^+$ Th1 and Th2 cell-lineage-specific transcription factors T-bet and GATA-3, respectively. In CD4$^+$ T cells, T-bet is rapidly and specifically induced in developing Th1 cells only and is a feasible candidate for involvement in the Th1-specific expression of the IFN-γ gene (49). On the other hand, reduction in GATA-3 expression in differentiated Th2 cells resulted in decreased production of IL-4 and IL-13 (54). High levels of GATA-3 instruct effector T cells to produce IL-4 and IL-13 by inducing characteristic DNase hypersensitivity sites in the IL-4 locus and IL-13 promoters (19, 22). Furthermore, GATA-3 is not known to influence IL-10 gene expression (39). Not only are T-bet and GATA-3 necessary for the development of CD4$^+$ Th1 and Th2 effector phenotypes, respectively, these lineage-specific transcription factors are prime candidates for the maintenance of cytokine memory from committed CD4$^+$ T helper cells (24). To simulate the in vivo condition of splenic CD4$^+$ T-cell in infected and treated mice, splenic T cells were stimulated with *L. donovani* promastigotes for 6 days, and RNA was isolated from MACS-purified CD4$^+$ T cells to study the expression profiles of GATA-3 and T-bet transcripts by real-time PCR. The extent of T-bet induction was found to be increased ~5-fold (Fig.4Ba), whereas an ~6-fold increase in GATA-3 transcription factor (Fig.4Bb) suggested the clonal expansion of both Th1 and Th2 subsets due to curative HCV therapy in consonance with substantial upregulation in IFN-γ, as well as IL-4 and IL-13, transcripts from splenic CD4$^+$ T cells. Our results also indicated that the observed surge in IFN-γ, IL-4, and IL-13 are indeed from *Leishmania*-reactive CD4$^+$ Th1 and Th2 clones generated in vivo and not due to artifactual in vitro skewing of CD4$^+$ T lymphocytes by rKMP stimulation.

Cytokines measurement at protein level in macrophage-depleted splenocytes reflected a similar representation with a >6-fold ($P < 0.001$) surge in IFN-γ production after HCV immunotherapy (Fig.4Ca), while Th2 cytokines IL-4 and IL-13 (Fig. 4Cc and d) were elevated by 4.2- and 2.74-fold, respectively. IL-10 showed >5.5-fold reduction in cured mice after HCV therapy (Fig.4Cb). CD8 depletion resulted in reduced expression of IFN-γ (2.19-fold), as well as IL-4 (1.88-fold) and

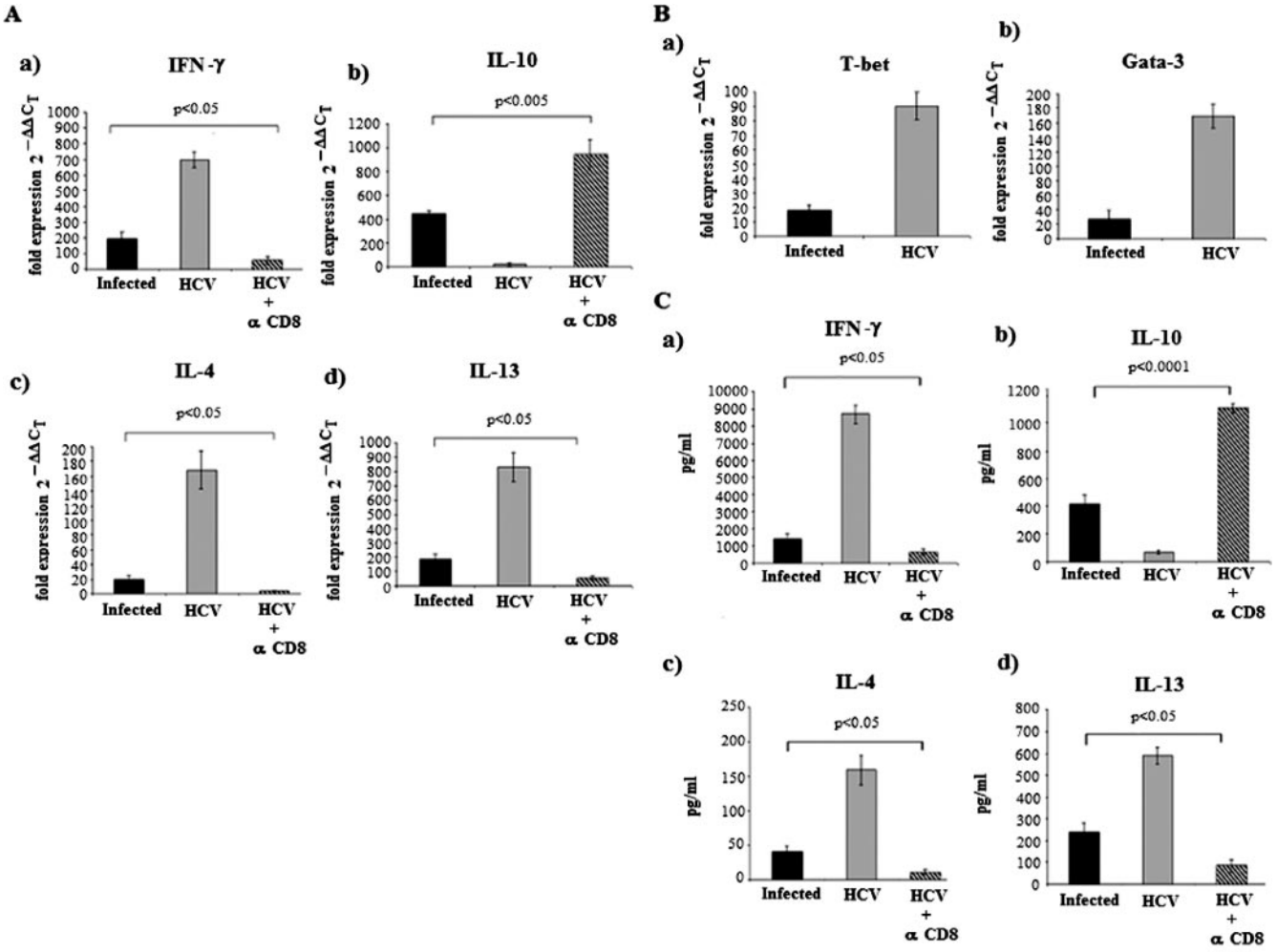

FIG. 4. (A) HCV therapy downregulates the IL-10 transcript from splenic $CD4^+$ T cells but not IL-4 and IL-13. Real-time PCR analysis was performed to detect the fold change in mRNA expression profiles of IFN-γ (a), along with the Th2 cytokines IL-10 (b), IL-4 (c), and IL-13 (d), in MACS-purified $CD4^+$ splenic lymphocytes (5×10^6/ml) of infected, HCV-treated mice (with or without depletion of $CD8^+$ T cells) versus normal BALB/c mice in response to 18 h of stimulation with rKMP-11 protein (5 μg/ml). The results are representative of eight individual mice per group analyzed separately, and the data represent the means ± the SE. The expression of a target gene was quantified by using the formula described in Materials and Methods. (B) HCV therapy significantly upregulates GATA-3 along with T-bet. The results of an analysis of the fold change in mRNA expression of the Th1 polarizing transcription factor T-bet (a) and the Th2 polarizing transcription factor GATA-3 (b) in splenic $CD4^+$ T lymphocytes of infected, HCV-treated mice versus normal BALB/c mice are shown. The results are representative of eight individual mice per group analyzed separately, and data represent the means ± the SE. (C) Only the IL-10 protein level is significantly reduced but Th1 cytokine IFN-γ and Th2 cytokines IL-4 and IL-13 are elevated after HCV therapy. ELISA measurements of the protein levels of IFN-γ (a), IL-10 (b), IL-4 (c), and IL-13 (d) were done with the supernatants of splenic lymphocytes (5×10^6/ml) depleted of macrophages from mice receiving therapeutic HCV treatment (with or without depletion of $CD8^+$ T cells) and from infected mice (four animals/group) stimulated with rKMP-11 protein (5 μg/ml). The results are representative of three experiments, and the data represent the means ± the SE.

IL-13 (1.48-fold), compared to infected mice. In accordance with transcriptional analysis, IL-10 protein level showed 2.65-fold elevated expression in CD8-depleted HCV-treated mice ($P < 0.0001$) with respect to infected controls. Compared to HCV-treated animals, CD8 depletion resulted in a >15-fold increase in IL-10 expression (Fig.4Cb). Although we did not compare IL-4, IL-13, and IL-10 mRNA expressions from purified $CD8^+$ T cells of splenic lymphocytes of HCV-treated mice with infected controls (the C_T values of these Th2 cytokine genes from $CD8^+$ T cells were consistently found to be >35 in both the groups), it is unlikely that the surge of IL-4 and IL-13 in HCV-treated mice is contributed by $CD8^+$ T cells. Nevertheless, it may be argued that the $CD8^+$ T-cell population present in macrophage-depleted splenocytes prepared for ELISA contributed to a >6-fold increased IFN-γ production after HCV therapy. These results along with cytokine transcript data suggested that IFN-γ, IL-4, and IL-13 protein levels were elevated from the *Leishmania*-reactive Th1 and Th2 clones in splenic lymphocytes of HCV-treated mice, whereas the IL-10 level was highly suppressed after immunotherapy. In the absence of activated $CD8^+$ T cells induced by HCV therapy, a suppressive effect of IL-10 is exerted on both Th1 and Th2 cytokines. Overall, the results suggest that the IL-10-rich splenic environment known to be created in the effector phase

of *L. donovani* infection is significantly downmodulated by HCV therapy.

DISCUSSION

Our study convincingly shows that hybrid cell immunotherapy, until now not implemented in the infectious disease model, cures *L. donovani*-infected BALB/c mice contingent on the generation of an antigen-specific $CD8^+$ CTL response. While cancer immunotherapy with HCV uses 3 to 25 doses of HCV to clinically stabilize patients with metastatic melanoma and breast and renal carcinoma (3, 50, 51), a single dose of HCV is sufficient to eliminate >80% of the parasites in late-stage *L. donovani* infection. In the context of the steady emergence of drug-resistant strains to all chemotherapeutic interventions, including the recently approved oral drug Miltefosine, the broad potential of HCV as a defined immunotherapeutic intervention cannot be ignored in future VL clinical trials. While $CD8^+$ T cells have gained importance in governing protective immunity in VL (34, 43), their association with Th2 cytokines has been related to the suppression of detrimental Th2 response in CL model (52). In our study, a decrease in or complete eradication of the parasite burden directly was correlated with HCV-induced generation of a strong antigen-specific $CD8^+$ CTL response. In a departure from the notion of the suppression of detrimental Th2 response by activated $CD8^+$ T cells observed in experimental CL, the Th2 cytokines IL-4 and IL-13 were not found to be suppressed by the elicitation of a vigorous anti-KMP-11-specific $CD8^+$ T-cell response and high IFN-γ production due to HCV therapy. Our finding is in agreement with clinical findings wherein both IFN-γ^+ and IL-4^+ $CD4^+$ T cells have been isolated from asymptomatic and cured VL patients (15, 25), as well as in agreement with experimental findings wherein an increased frequency of IFN-γ- and IL-4-producing splenic and hepatic cells was associated with a decreased *L. donovani* burden as a result of curative anti-CTLA monoclonal antibody therapy (31). Our observations also strongly indicated that IL-10 was the only Th2 cytokine suppressed by activated $CD8^+$ T cells after HCV therapy. Our findings also support a previous report on patients with active VL, in which the transcript level of IL-10 was significantly elevated but eventually decreased markedly with the resolution of the disease (16). The role of the Tc2 population of $CD8^+$ T cells as potential sources of IL-4, IL-13, and IL-10 was ruled out since the purified $CD8^+$ T cells of HCV-treated mice did not show detectable transcripts of these three cytokines (C_T [threshold cycle] > 35) (data not shown). Curiously, the production of IFN-γ, as well as the production of IL-4 and IL-13, was significantly diminished by $CD8^+$ T-cell depletion in HCV-treated mice, whereas IL-10 production was greatly enhanced. Hence, the strong association of a curative response found with the suppression of IL-10, and not other Th2 cytokines, with HCV-mediated therapy points to the specific inhibition of IL-10 by activated $CD8^+$ T cells. It therefore might also be reasonable to argue that the suppression of Th2 cytokines by $CD8^+$ T cells might principally involve the suppressive activity of IL-10-producing T-cell subsets. Besides being a potent suppressor of all known microbicidal effector functions of macrophages, IL-10 can also negatively regulate both Th1 and Th2 response to antigen (13). Studies in mice (30) and humans (16) indicate that IL-10 is the major immunosuppressive cytokine in VL. Moreover, it has been pointed out that in human VL, IL-10 is not produced from classical Th2 cells (16). Thus, in *L. donovani* infection IL-10 is increasingly being designated as the suppressive cytokine produced from a population of regulatory T cells (8); expansion and/or activation of these cells might be able to suppress both Th1 and Th2 cells. Reportedly, in *L. donovani* infection, an IL-10-rich splenic environment is created during the effector phase of the antileishmanial immune response by IL-10-secreting $CD25^+$ suppressor T cells that inhibit the activation of naive T cells (8). Interestingly, it has been recently shown that IFN-γ produced by tumor-specific $CD8^+$ CTL cells inhibits the generation and/or activation of suppressive regulatory T cells (35). Hence, it is worth speculating that vaccine-induced activated $CD8^+$ T cells are able to downregulate IL-10 that, in turn, resulted in the release of both Th1 and Th2 cells to produce IFN-γ from the former cell population and IL-4 and IL-13 from the latter cell population. Alternatively, it also remains a distinct possibility that HCV therapy may also expand preexisting, antigen-specific $CD8^+$ T cells by directly or indirectly releasing them from suppressive control by T regulatory (Treg) cells. In the present study, the T-cell anergy in response to SLA in infected cells that become responsive to antigen after HCV therapy and its subsequent reversion to the anergic state after $CD8^+$ T-cell depletion might be governed by the production of a suppressive cytokine such as IL-10. Finally, reduced expression of both T-bet and GATA-3 from infected cells and elevated levels of these transcription factors in $CD4^+$ T splenic T cells of cured mice confirmed expansion of *Leishmania*-reactive Th1 and Th2 clones. In fact, elevated levels of GATA-3, a master transcriptional regulator of the IL-4 and IL-13 locus from $CD4^+$ T cells, corroborated well with increased IL-4 and IL-13 production in HCV-treated mice. Importantly, in light of a recent report that suggested the failure of GATA-3 to regulate transcriptional activity of IL-10 gene in vivo (39), transcription of IL-10 might not be contingent on the GATA-3 activity of Th2 cells if the principle source of IL-10 is from a discrete population of $CD4^+$ T-cell subset. Further studies are required to distinguish between the contributory roles of IL-4- and IL-13-producing Th and IL-10-producing Treg cells in infection- and vaccine-induced immune response.

Early studies in both mice (17) and humans (18) suggested that cure was independent of the differential production of Th1 and Th2 cytokines. Although IFN-γ has been universally regarded as a host-protective cytokine in both CL and VL, a disease-promoting role for an IL-4- and IL-13-associated Th2 response in VL has not been demonstrated. Consequently, studies in B6/129 (36) and BALB/c (42) mice have shown that IL-4- and IL-4Rα-deficient animals are in fact more susceptible to disease than their wild-type counterparts. In addition, not only is IL-4 and IL-4Rα signaling essential for optimal clearance of *L. donovani* from the liver (42), it is also essential for effective T-cell-dependent chemotherapy (2) and vaccine-induced resistance mediated by $CD8^+$ T cells (41). In the absence of IL-4, an IFN-γ-producing Th1 response cannot be maintained after chemotherapy or cannot be induced by vaccination. Hence, additionally beneficial, protective roles for IL-4 and IL-13, along with IFN-γ, in *L. donovani* infection cannot be discounted. Furthermore, a cure in susceptible

BALB/c mice is largely dependent on IL-12 that promotes the expansion of both the Th2 and the Th1 response (10). As several studies have suggested, a protective influence for IL-4 and IL-13 in leishmaniasis, as well as beneficial roles for IL-4 and IL-13, along with IFN-γ, in *L. donovani* infection, cannot be discounted (2, 36, 41). Although these data indicate that the essential effector mechanism of HCV is mediated by $CD8^+$ T cells, this doesn't exclude the beneficial role of $CD4^+$ T cells. $CD8^+$ CTL activation by $CD4^+$ T cells can also be vital, since $CD4^+$ T cell help has been shown to be required at the priming phase of CTLs (38).

The present study has shown that hybrid cell therapy capable of generating a vigorous antigen-specific $CD8^+$ CTL response by exploiting the allogeneic MHC response can be implemented in future vaccine designs against an advanced state of VL. Successful application of HCV in this infectious disease model opens up an avenue for its future use as a combined therapeutic approach against multiple infectious diseases with defined immunodominant antigens.

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