DNA-Encoded Fetal Liver Tyrosine Kinase 3 Ligand and Granulocyte Macrophage-Colony-Stimulating Factor Increase Dendritic Cell Recruitment to the Inoculation Site and Enhance Antigen-Specific CD4+ T Cell Responses Induced by DNA Vaccination of Outbred Animals

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DNA-based immunization is a contemporary strategy for developing vaccines to prevent infectious diseases in animals and humans. Translating the efficacy of DNA immunization demonstrated in murine models to the animal species that represent the actual populations to be protected remains a significant challenge. We tested two hypotheses directed at enhancing DNA vaccine efficacy in outbred animals. The first hypothesis, that DNA-encoding fetal liver tyrosine kinase 3 ligand (Flt3L) and GM-CSF increases dendritic cell (DC) recruitment to the immunization site, was tested by intradermal inoculation of calves with plasmid DNA encoding Flt3L and GM-CSF followed by quantitation of CD1+ DC. Peak DC recruitment was detected at 10–15 days postinoculation and was significantly greater (p < 0.05) in calves in the treatment group as compared with control calves inoculated identically, but without Flt3L and GM-CSF. The second hypothesis, that DNA encoding Flt3L and GM-CSF enhances immunity to a DNA vector-expressed Ag, was tested by analyzing the CD4+ T lymphocyte response to Anaplasma marginale major surface protein 1a (MSP1a). Calves immunized with DNA-expressing MSP1a developed strong CD4+ T cell responses against A. marginale, MSP1a, and specific MHC class II DR-restricted MSP1a epitopes. Administration of DNA-encoding Flt3L and GM-CSF before MSP1a DNA vaccination significantly increased the population of Ag-specific effector/memory cells in PBMC and significantly enhanced MSP1a-specific CD4+ T cell proliferation and IFN-γ secretion as compared with MHC class II DR-matched calves vaccinated identically but without Flt3L and GM-CSF. These results support use of these growth factors in DNA vaccination and specifically indicate their applicability for vaccine testing in outbred animals. The Journal of Immunology, 2002, 169: 3837–3846.

Vaccination using DNA-encoded Ags induces cellular and humoral immunity against microbial pathogens (1–3). Cellular uptake and intracellular expression of the vaccine DNA allows prolonged expression of Ag, mimicking the effect of a live vaccine (4, 5). In addition to improved Ag delivery, DNA vaccine vectors can be modified to enhance the early stages of Ag processing, presentation, and priming of Ag-specific B and T lymphocytes. Modifications to the vector itself, such as inclusion of stimulatory CpG motifs (6), or insertion of cytokine genes to be coexpressed with the vaccine Ag have been shown to dramatically enhance MHC class I- (7) and class II- (8) dependent immune responses and confer protection against virulent challenge (9). Unsurprisingly, the immunogenicity of DNA vaccines and the ability of modified vectors to enhance immunity have been tested primarily in mice. Translating results from these murine models to the outbred species that represent the actual populations to be protected by vaccination is a significant barrier to deployment of DNA vaccines. In vivo studies in humans and cattle have demonstrated that, in contrast to the studies in murine models, multiple inoculations of high doses of DNA vaccine are required to induce significant immune responses (10–12). In addition, these studies revealed that individual vaccinates are low responders, even when they carry the MHC alleles capable of presenting the DNA vaccine-encoded epitopes (10–12). Consequently, identifying DNA vaccine modifications that enhance immunogenicity in outbred species is a high priority.

We chose to focus on enhancing early events in Ag processing and presentation. Increasing DC recruitment and activation at the immunization site has been shown to enhance vaccine immunogenicity in both murine models (8, 9) and outbred species (13), including humans (14). Specifically, administration of the hematopoietic growth factor fetal liver tyrosine kinase 3 ligand (Flt3L) alone or in combination with GM-CSF significantly expands the number of dendritic cells (DC) in both mice and humans (15–20). Testing of DNA vaccine vectors encoding Flt3L or, separately,

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3 Abbreviations used in this paper: Flt3L, fetal liver tyrosine kinase ligand; DC, dendritic cell; MSP, major surface protein; MSP1aED, modified extracellular domain of MSP1a; FL, Florida strain; MBP, maltose-binding protein; URBC, uninfected bovine erythrocyte; SI, stimulation index.
GM-CSF, has been shown to enhance Ag-specific immunity in mice (8, 9, 21–23). However, comparative experiments testing the protection induced by a DNA vaccine encoding the Plasmodium yoelli circumsporozoite protein revealed significant enhancement by GM-CSF only in mice (9) and not in rhesus monkeys (24). To date, the efficacy of DNA vaccines encoding Flt3L has not been tested in any species other than mice (25, 26). The goal of the present study was to test whether Flt3L and GM-CSF, incorporated into a DNA vaccine vector, would increase DC recruitment and result in enhanced Ag-specific immune responses, using calves as the outbred species. Administering a combination of recombinant Flt3L and GM-CSF proteins to mice has an additive effect on the overall number of DC and Ag-capture efficiency is augmented in DC generated using the combined cytokines (20). The ability of DNA-encoded Flt3L and GM-CSF to increase DC recruitment to the immunization site and to enhance Ag-specific immune responses has not been reported for any animal species.

To test whether DNA-encoded Flt3L and GM-CSF would enhance immunity against a DNA vaccine-encoded Ag, the gene encoding the major surface protein (MSP-1a) of the rickettsia Anaplasma marginale was selected (27, 28). A. marginale is the most prevalent tick-borne pathogen of cattle worldwide and there are currently no safe and effective vaccines available (29, 30). Immunity against A. marginale is associated with a MHC class II-restricted CD4+ T cell response and MSP1a is targeted by the protective response (31, 32). Importantly, a CD4+ T cell epitope-rich region in MSP1a has been identified (aa 251–366) and specific MHC class II DR-restricted epitopes have been localized (52). This mapping allows testing whether the Flt3L/GM-CSF treatment enhances T cell responses to specific epitopes in vaccines with the appropriate MHC class II alleles. In this manuscript, we report testing the following hypotheses: 1) DNA-encoding Flt3L and GM-CSF increases DC recruitment to the immunization site; and 2) DNA-encoding Flt3L and GM-CSF enhances CD4+ T cell responses to MSP1a expressed in a DNA vaccine vector.

Materials and Methods

Plasmid construction and purification

Generation of a construct expressing bovine Flt3L has been described previously (33). To generate a construct expressing GM-CSF, the open-reading frame encoding bovine GM-CSF was PCR-amplified from pTargetGM-CSF (34). The forward primer (5’-ATAGATCATCATGGCCTG CAGAACCCTGCTCCTCC-3’) introduced an EcoRV restriction site (in italics) at the 5’ end and the reverse primer (5’-TATGGATCATCCTACT TCTGGGGCTGTTCCAGGC-3’) introduced a BamHI restriction site (in bold) at the 3’ end. The EcoRV-BamHI fragment was ligated into EcoRV-BamHI digested VR-1055 to generate pEcoRV-BamHI digested VR-1055 eukaryotic expression vector (Vical, San Diego, CA) to generate pVRG-CSF.

Two constructs, one encoding the full-length MSP1a and the other encoding a modified extracellular domain of MSP1a (MSP1aED) were generated. The open-reading frame encoding the full-length MSP1a was released from pRC/CMV/MSP1a (11) by digestion with Prl and ligation into the Prl-digested VR-1055. A construct containing the mps1a gene in the correct orientation was designated pVRMSP1a. To improve MSP1aED expression efficiency, the gene encoding MSP1aED was modified to increase hydrophilicity and a secretion signal sequence was added. To increase MSP1aED hydrophilicity, the MSP1a hydrophilic point was modified by using the algorithm of Hofmann (www.chembio.northwestern.edu/TMPRED_form.html) and a predicted membrane insertion domain (residues 153–158) was deleted. To direct MSP1aED secretion, the human CD5 secretory signal sequence was added (35). The open-reading frame encoding the MSP1aED was PCR-amplified from a recombinant plasmid (pVR1) containing a genomic DNA fragment of A. marginale (Virginia strain) mps1a gene (27). The forward primer (5’-TACTGTGTAGTAGTGTGTC GGATCC-3’) and the reverse primer (5’-TTAGTACATCCTGCTGTTGTC CGAAGG-3’) introduced a BamHI restriction site (in italics) at the 3’ end. The CD5 signal sequence was PCR-amplified and the product was sub-cloned into the PCR-Blunt vector (Invitrogen, Carlsbad, CA) to generate pCD5ss. The MSP1aED gene was ligated inline as a Prl-BamHI fragment into Prl-BamHI-digested pCD5ss to generate pCD5ssMSP1aED. The CD5ssMSP1aED open-reading frame, encoding the modified MSP1aED (MSP1aEDm), was released as an EcoRV-BamHI fragment and was ligated into EcoRV-BamHI digested VR-1055 to generate pVRMSP1aEDm.

The pVRFlt3L, pVRGM-CSF, pVRMSP1a, and pVRMSP1aEDm constructs, as well as the unmodified VR-1055 vector, were amplified in DH5α Escherichia coli cells (Life Technologies, Gaithersburg, MD) and large-scale plasmid DNA was purified using an endo-free Plasmid Gigaprep kit (Qiagen, Valencia, CA). The endotoxin content in the plasmid DNA preparations was measured using a Limulus Amebocyte Assay kit (BioWhitaker, Walkersville, MD) and was <5 EU/mg in all samples.

Protein expression

Flt3L, GM-CSF, MSP1a, and MSP1aEDm were expressed in COS-7L cells (Life Technologies) as previously described (33). The pVRFlt3L-transfected COS-7L cell monolayer was incubated with a 1/200 dilution of a murine anti-bovine Flt3 ligand peptide antiserum (33) in blocking buffer, whereas the pVRGM-CSF-transfected COS-7L cell monolayer was incubated with 5 μg/ml anti-bovine GM-CSF 20.1 mAb (VMRD, Pullman, WA). The pVRMSP1a- and pVRMSP1aEDm-transfected COS-7L cell monolayers were incubated with 5 μg/ml anti-MSP1a mAb Ana22B1 (27). Duplicate plates were either reacted with a negative control peptide antiserum or isotype control mAb. Following washes in blocking buffer, the monolayers were incubated with a 1/2500 dilution of alkaline phosphatase-conjugated caprine anti-murine mAb (Tropix, Bedford, MA) in blocking buffer. Following washes in blocking buffer, the alkaline phosphatase activity was detected using Fast Red TR/Naphthol AS-MX substrate (Sigma-Aldrich, St. Louis, MO). Stained cells were visualized and photographed using an inverted phase contrast microscope model CK-2 (Olympus Optical, Tokyo, Japan). The microscopy was also used to count pVRMSP1a- and pVRMSP1aEDm-transfected COS-7L cells expressing the encoded Ag and the counts were used to calculate expression efficiency.

To generate protein for biological assays or T cell proliferation assays, COS-7L cell monolayers were transfected with the DNA constructs as above and 1 day posttransfection, the transfection medium was replaced with serum-free medium VP-SFM (Life Technologies) supplemented with 10% FCS and allowed to incubate for 7 days. Supernatants, serial dilutions of the supernatants were used to generate dot blots that were then reacted with peptide-specific antisera or mAb.

Bioassays for Flt3L and GM-CSF

Biological activity of the COS-7L-expressed bovine Flt3L has been reported previously (33). The COS-7L-expressed bovine GM-CSF was tested for biological activity using PBMC from a neonatal calf. Briefly, PBMC (1 × 10^6 cells per well) were incubated in triplicate with serum-free medium VP-SFM (Life Technologies) supplemented with 4 mM t-glutamine. Supernatants were harvested 96 h posttransfection and concentrated 10-fold using a filter device with a 10-kDa molecular mass cut-off (Millipore, Bedford, MA). To verify protein expression in the concentrated supernatants, serial dilutions of the supernatants were used to generate dot blots that were then reacted with peptide-specific antisera or mAb.

Induction of MSP1-specific CD4+ T cell recall responses with MSP1aEDm Ag

Induction of MSP1-specific T cell recall responses by the COS-7L-expressed pVRMSP1aEDm Ag was verified by lymphocyte proliferation assays as described previously (31). Briefly, T cell lines were generated from calves immunized with native MSP1 were incubated in duplicate with serial dilutions of the pVRMSP1aEDm COS-7L supernatant and 2 × 10^7 irradiated autologous PBMC for 3 days. rMSP1a carboxyl region (0.2–10.0 μg/ml; Ref. 31) was used as a positive control and supernatant from the COS-7L cells transfected with the VR-1055 vector was used as a negative control. The cells were radiolabeled for the last 18 h of culture with 0.25 μCi/ml ^3H]thymidine for the last 4 h and harvested using an automated cell harvester (Tomtec, Orange, CT), and counted with a liquid scintillation counter. The mean ^3H]thymidine incorporation (cpm ± 1 SD) was plotted against supernatant dilutions.

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Immunization of calves

Six male Holstein calves (7-mo-old) seronegative for *A. marginale* were used in this study. PBMC from these calves were tested in a proliferation assay with *A. marginale* Ags and found to be nonresponsive. MHC class II-DRB3 alleles were defined by PCR-restriction fragment length polymorphism analysis of exon 2 (36). The DRB3 alleles are as follows: for calf 18, DRB3*24/*24, for calf 19, DRB3*16/*22, for calf 20, DRB3*16/*22, for calf 21, DRB3*16/*24, for calf 23, DRB3*16/*24, and for calf 24, DRB3*22/*24. The calves were allocated to three groups and inoculated with 1 mg of each DNA construct or vector following the protocol summarized in Table I. For each dose, multiple intradermal injections (200 μl with 1 mg of each DNA construct or vector following the protocol summarized in Table I). The calves were inoculated with 1 mg of each DNA construct or vector following the protocol summarized in Table I.

DC recruitment

Recruitment of DC to the skin was analyzed by immunohistochemistry using cryosections generated from biopsies taken randomly from the immunization site. On day 1, preinoculation biopsies were taken from the left flank region before DNA injection on the right flank region. Postinoculation biopsies were taken from the right flank immunization site on days 5, 10, 15, and 20 using a 6-mm Uni-Punch Disposable Biopsy Punch (Premier Medical Products, King of Prussia, PA). The skin biopsies were snap-frozen and stored at −80°C. Cryostat sections (4–6 μm) were cut from the frozen skin biopsies and sections (four per slide) were collected on charged glass slides (Probe On Plus; Fisher, Pittsburgh, PA). Three slides were generated from each biopsy, fixed in acetone for 10 min and air-dried for 10 min at room temperature. The sections were probed with 1 μg/ml THP7α mAb (Monoclonal Ab Center, Washington State University, Pullman, WA) that recognizes the bovine orthologue of the CD1 determinant strongly expressed on skin DC (37). An isotype-matched mAb and secondary Ab were included as negative controls. Following washes with PBS, the slides were incubated with a ready-to-use Biotinylated Murine Linking Reagent (Signet Laboratories, Dedham, MA), washed again and incubated with 1/10,000 dilution of 2 mg/ml alkaline phosphatase conjugate (Life Technologies). Following washes with PBS, the alkaline phosphatase activity was detected using Fast Red TR/Naphthol AS-MX substrate (Sigma-Aldrich). The slides were washed with distilled water and counterstained with hematoxylin. Stained skin sections were photographed using a Zeiss Axioskop microscope (Zeiss, Jena, Germany) whereas CD1+ DC (with nuclei) were visualized and counted using a light microscope (magnification, ×40) fitted with an ocular grid. From each slide, DC were counted in the superficial and deep dermis from an area of 1.28 mm2 and the mean ± 1 SD of the CD1+ cells from the three slides generated per biopsy was calculated. Only CD1+ cells with clearly visible nuclei were counted. The significance of the difference between means of the Flt3L/GM-CSF-treated and the control groups, and the variation of the means over time was analyzed by ANOVA followed by the Bonferroni pairwise multiple comparison test. A value of *p* < 0.05 was considered significant.

Establishment of T lymphocyte lines

Short-term *A. marginale*-specific T lymphocyte lines were established from calves immunized 13-mo postimmunization as described previously (11, 31). Briefly, CD8+ and γδ T lymphocytes were depleted by incubating PBMC with CD8-specific mAb 7C2B and γδ TCR-specific mAb GB21A (Monoclonal Ab Center, Washington State University), followed by immunomagnetic separation using sheep anti-mouse IgG-coated Dynabeads (Dynal Biotech, Lake Success, NY) as described (38). T lymphocyte lines were established from the CD8+ γδ+ PBMC by stimulation with homogenate prepared from the Florida (FL) strain of *A. marginale*. Briefly, 4 × 10^6 CD8+ γδ+ PBMC per well in 24-well plates (Costar, Cambridge, MA) were cultured for 7 days in a volume of 1.5 ml of complete RPMI 1640 medium (31) with 10 μg/ml *A. marginale* FL strain homogenate. Background proliferation was lowered by subculturing cells, without Ag, to a density of 7.5 × 10^5 cells/well and cultured with 2 × 10^5 irradiated (3000 rad) autologous PBMC as a source of APCs. The T lymphocyte lines were maintained for 7 more days and then assayed for Ag-dependent proliferation.

Cell surface phenotypic analysis

Differential markers on T lymphocyte lines were analyzed by indirect immunofluorescence and flow cytometry as described (39). Bovine CD2, CD3, CD4, CD8, CD14, CD21, and the γ-chain of the γδ TCR-specific mAbs used have been described previously (31).

Proliferation assays

Proliferation assays were conducted as described (11, 31). Briefly, T lymphocyte lines (3 × 10^5 cells) were cultured in triplicate wells of round-bottom 96-well plates (Costar) for 3 days in a total volume of 100 μl of complete medium containing Ag and 2 × 10^5 irradiated autologous PBMC as APC. Ags consisted of *A. marginale* FL strain homogemate (0.4 to 10.0 μg/ml), rMSP1a carboxy region (residues 242–540) expressed as a maltose-binding protein (MBP) fusion protein (0.4 to 10.0 μg/ml) (31), and the COS-7L-expressed MSP1aEd1Ed2 (1/100/1/250 dilution) peptides F2.1-F2.5 and F3.1-F3.6 (0.4–10.0 μg/ml) were also included to test whether the T lymphocyte lines recognize MSP1a CD4+ T cell epitopes defined by CD4+ T cell lines and clones generated from calves immunized with native MSP1 (52). The F2 and F3 peptides (30 mer overlapping with 10 residues) span the F2 residues 334–444 and F3 residues 424–540. MSP2 (residues 112–244) expressed in the extracellular domain of MSP1a (27). These regions were defined by dividing residues 242–540 into three fragments (Flt3–F3) of 112 residues. COS-7L-expressed F2 and F3 proteins induced MSP1a-specific recall responses and thus, F2 and F3 peptides were used to map T cell epitopes within these regions (52). Membranes prepared from uninfected bovine erythrocytes (URBC), rMBP (New England Biolabs, Beverly, MA), the supernatant derived from uninfected COS-7L cells transfected with the VR-1055 vector, and peptide MSP2 P1 derived from the unrelated *A. marginale* MSP2 (40) were used as negative control Ags. Cells were radiolabeled, harvested, and counted as above. Results are presented as the stimulation index (SI), defined as the mean cpm of triplicate cultures of cells plus Ag divided by the mean cpm of triplicate cultures of cells plus medium. The significance of the differences in proliferation of T lymphocytes from the Flt3L/GM-CSF plus Ag-treated and control groups was analyzed by Student’s t-test using cpm values. A value of *p* < 0.05 was considered significant.

Detection of IFN-γ in supernatants of T lymphocyte lines

Short-term *A. marginale*-specific T lymphocyte lines were established as described above and then were restimulated for 72 h with autologous APC and *A. marginale* FL strain homogenate (10 μg/ml), rMSP1a carboxy region-MBP fusion protein (10 μg/ml), and the MSP1aEd COS-7L supernatant (1/50 dilution); supernatants were tested for IFN-γ production by ELISA. Controls consisted of supernatants from cell lines stimulated with membranes prepared from uninfected bovine erythrocytes (10 μg/ml), rMBP (10 μg/ml), and the VR-1055 COS-7L supernatant (1/50 dilution). The bovine IFN-γ assay was performed using an ELISA kit (BOVIGAM; CSL, Parkville, Victoria, Australia) according to the manufacturer’s protocol. The IFN-γ activity in culture supernatants diluted 1/4–1/100 was determined by comparing with a standard curve obtained as described previously (39). The results are presented as units of IFN-γ per milliliter. The significance of the differences in units of IFN-γ per milliliter secreted by T lymphocyte lines was analyzed by ANOVA followed by the Bonferroni pairwise multiple comparison test. A value of *p* < 0.05 was considered significant.

Table I. DNA immunization protocol

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<td>VR-1055</td>
<td>MSP1aEd1Ed2</td>
</tr>
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*a MHC class II-DRB3 alleles were defined by PCR-restriction fragment length polymorphism analysis of exon 2 (36).

*b Calves were inoculated intradermally with DNA constructs encoding Fli3L and GM-CSF or vector alone.

c Calves were inoculated with DNA construct encoding Ag or vector alone at the same site where the cytokine constructs were injected.

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lymphocyte lines established from the Flt3L/GM-CSF plus Ag-treated and the control groups was analyzed by Student’s t test.

Detection of Ag-specific lymphocytes by ELISPOT

To detect MSP1α-specific T lymphocytes without in vitro expansion, IFN-γ-expressing cells in PBMC were quantified using an ELISPOT assay 13-mo postvaccination. Hybridomas CC302 and CC330 were derived from BALB/C mice immunized with bovine IFN-γ using the protocol described by Kwong et al. (41) for IL-10. Briefly, mice were inoculated three times i.m. with plasmid-encoding bovine IFN-γ and boosted just before removal of spleens with the same plasmid (CC330) or with recombinant bovine IFN-γ protein (CC330) given i.p. The ELISPOT assays were conducted in triplicate wells of MultiScreen-HA plates (Millipore). The wells were coated with mouse anti-bovine IFN-γ mAb CC330 (0.8 μg/ml) in distilled water and, after incubation for 2 h at room temperature, excess Ab was removed by washing with PBS containing 0.05% Tween 20 (PBST). The coated wells were blocked with PBS containing 1% BSA for 1 h at room temperature. The plates were then washed with PBST and incubated at room temperature with complete RPMI until cells were added. Freshly isolated PBMC (5 × 10⁶ or 1 × 10⁶ cells/well) were added to each well in 50-μl volumes. Ags or control mitogens were then added in 50-μl volumes. MSP1αED₅₅ was tested using supernatants of pVRMSP1αED₅₅-transfected COS-7L cells at a dilution of 1/40. PHA-L at a 1 μg/ml final concentration plus 0.01 ng/ml recombinant human IL-12 plus 0.5 ng/ml recombinant human IL-18 were used as a positive control as this combination has been shown to stimulate high levels of IFN-γ in bovine PBMC.

FIGURE 1. A, Expression of bovine Flt3L in COS7L cells. Monolayers of COS-7L cell transfectants were assayed for Flt3L expression using a murine anti-bovine Flt3L peptide serum by in situ immunocytochemistry. Aa and Ab, COS-7L cells transfected with the pVRFlt3L construct encoding bovine Flt3L; Ac, COS-7L cells transfected with empty vector; and Ad, COS-7L cells. Aa, Ac, and Ad COS-7L cell monolayers were probed with the murine anti-bovine Flt3L peptide serum, whereas Ab was probed with a negative control peptide antiserum (murine anti-Babesia bigemina rhoptry-associated protein-1c peptide antiserum). B, Expression of bovine GM-CSF in COS7L cells. COS-7L cell transfectants were assayed for GM-CSF expression using a murine anti-bovine GM-CSF mAb (GM-CSF 20.1) by in situ immunocytochemistry. Ba and Bb, COS-7L cells transfected with the pVRGM-CSF construct encoding bovine GM-CSF; Bc, COS-7L cells transfected with empty vector; and Bd, COS-7L cells. Ba, Bc, and Bd COS-7L cell monolayers were probed with mAb GM-CSF 20.1, whereas Bb was probed with an isotype-matched negative control mAb. C, Expression of full-length A. marginale MSP1α and MSP1αED₅₅ in COS-7L cells. COS-7L transfectants were assayed for MSP1α and MSP1αED₅₅ expression using Ana22B1 mAb by in situ immunocytochemistry. Ca andCb, COS-7L cells transfected with the pVRMSP1α construct encoding full-length A. marginale MSP1α (Virginia strain); Ce and Cf, COS-7L cells transfected with the pVRMSP1αED₅₅ construct encoding MSP1αED₅₅, Cc and Cg, COS-7L cells transfected with empty vector; Cd and Ch, COS-7L cells. Ca, Cc–Ce, Cg, and Ch COS-7L cell monolayers were probed with the Ana22B1 mAb, whereas Cb and Cf were probed with an isotype-matched negative control mAb.
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FIGURE 2. COS-7L expressed MSP1aEDm Ag induces a CD4+ T cell recall response by T cells from native MSP1-immunized calves. Induction of MSP1-specific T cell recall responses by the COS-7L-expressed MSP1aEDm Ag was verified by lymphocyte proliferation assays using MSP1-specific CD4+ T cell clones. A, clone 87.2A1; B, clone 93.4E4. ○, COS-7L-expressed MSP1aEDm Ag; ◊, supernatant from COS-7L cells transfected with the VR-1055 vector. Each point represents the mean of [3H]thymidine incorporation in cpm from three wells ± SD.

Results
Plasmid DNA expression of Flt3L and GM-CSF
Protein expression of Vical 1055-encoded Flt3L and GM-CSF transfected into COS-7L cells was assayed by in situ immunocytochemistry. Expression was detected in the pVRFlt3L and pVRGM-CSF COS-7L cell transfectants but not the respective negative controls (Fig. 1, A and B). Biological activity of the COS-7L-expressed bovine GM-CSF was tested for biological activity using PBMC. The supernatant from COS-7L cells expressing the bovine GM-CSF, but not the supernatant from COS-7L cells transfected with the VR-1055 vector, stimulated a dose-dependent lymphocyte proliferative response (maximal SI = 63, data not shown).

Plasmid DNA expression of MSP1a and MSP1aEDm
Immunocytochemical detection using mAb Ana22B1 revealed expression in COS-7L cells transfected with either pVRMSP1α or pVRMSP1αEDm (Fig. 1C). Analysis of protein expression efficiency (total number of cells expressing protein/total number of transfected cells × 100) showed that the pVRMSP1α COS-7L cell transfectants had an expression efficiency of <1%, whereas the pVRMSP1αEDm transfectants consistently had an expression efficiency >50% (Fig. 1C; data not shown). Dot blot analysis of concentrated supernatants generated from the COS-7L cell transfectants showed that MSP1aEDm was secreted but no MSP1a was detected in supernatant generated from COS-7L cells transfected with the pVRMSP1α construct (data not shown).

The COS-7L-expressed MSP1aEDm Ag, but not the supernatant from COS-7L cells transfected with the VR-1055 vector, induced dose-dependent Ag-specific proliferation of CD4+ T cell clones generated from calves immunized with native MSP1 (Fig. 2). These CD4+ T cell clones also proliferated in a dose-dependent manner to purified MSP1α (clone 87.2A1, maximal SI = 50.7, and clone 93.4E4, maximal SI = 22.2). This result shows that the MSP1αEDm Ag stimulates CD4+ T cells primed using native MSP1, and therefore bears epitopes represented on the native protein. Consequently, the pVRMSP1αEDm construct was chosen for DNA immunizations.

Coadministration of DNA-encoded Flt3L and GM-CSF enhances DC recruitment to a cutaneous inoculation site
Constructs expressing Flt3L and GM-CSF were coadministered intradermally following the protocol summarized in Table I. DC recruitment was analyzed by immunohistochemistry on biopsy specimens using anti-CD1 mAb TH97A. There was an increase in

FIGURE 3. Flt3L and GM-CSF enhance DC recruitment to the skin. Recruitment of DC to the skin was analyzed by quantitative immunohistochemistry using anti-CD1 mAb TH97A. A, Cryostat section from a preinoculation biopsy probed with mAb TH97A; B, cryostat section from a day 10 biopsy probed with mAb TH97A; C, cryostat section from a day 10 biopsy probed with an isotype-matched negative control mAb; and D, cryostat section from a day 10 biopsy probed with secondary biotin-labeled caprine anti-murine mAb.
CD1+ DC following treatment with the Flt3L/GM-CSF constructs (Fig. 3). To demonstrate the kinetics of DC recruitment at the cutaneous site after the treatment, the means of DC counts from skin biopsies taken at various time points were calculated. Peak numbers of DC at the inoculation site in Flt3L/GM-CSF-treated calves were significantly greater (p < 0.05) than those in control calves or preinoculation biopsies (Fig. 4). Importantly, there were no statistically significant increases in the numbers of skin DC at the inoculation site of calves vaccinated with the VR-1055 vector (data not shown). The MSP1a-specific proliferative responses of CD4+ T cell lines from the Flt3L/GM-CSF-treated and MSP1aEDm-immunized calves pretreated with Flt3L/GM-CSF were significantly higher than those of MSP1aEDm-immunized calves that did not receive Flt3L/GM-CSF (p < 0.05). The responses of CD4+ T cell lines from the MSP1aEDm-immunized calves that did not receive Flt3L/GM-CSF were significantly greater (p < 0.05) than those of CD4+ T cell lines from the negative controls.

The ability of Flt3L and GM-CSF to increase the number of Ag-specific effector/memory cells was tested by an IFN-γ ELISPOT assay using both 5 × 107 and 1 × 106 PBMC per well. The number of MSP1a-specific cells in MSP1aEDm-immunized calves pretreated with Flt3L/GM-CSF was significantly higher (p < 0.05) than those of MSP1aEDm-immunized calves that did not receive cytokines (Table II). Pairwise comparison between animals with identical DR haplotypes in these two groups, B0019 with B0021 (both are 16/22) and B0023 (both are 16/24), B0018 with B0023 (both are 16/24), also demonstrated significantly higher (p < 0.05) numbers of MSP1a-specific effector/memory cells in the MSP1aEDm-immunized calves pretreated with Flt3L/GM-CSF (Table II).
The CD4⁺ T cell lines were also tested for their ability to recognize MSP1a CD4⁺ T cell epitopes defined by CD4⁺ T cell lines and clones generated from calves immunized with native MSP1a (52). The CD4⁺ T cell lines established from calves treated with Flt3L/GM-CSF before MSP1aEDₘ immunization had significantly higher proliferative responses to the MSP1a peptides than CD4⁺ T cell lines established from the calves immunized with MSP1aEDₘ alone (Table III). These data confirmed that the CD4⁺ T cells induced by MSP1aEDₘ recognize epitopes that also stimulate T cell responses upon native MSP1 immunization.

Flt3L and GM-CSF enhance IFN-γ production by MSP1a-specific CD4⁺ T cell lines

The ability of Flt3L and GM-CSF to augment IFN-γ production by MSP1a-specific CD4⁺ T cells was demonstrated by analyzing supernatants from Ag-stimulated short-term MSP1a-specific CD4⁺ T cell lines. Significantly more (p < 0.05) IFN-γ was produced by CD4⁺ T cell lines established from the Flt3L/GM-CSF-pretreated and MSP1aEDₘ-immunized calves compared with that produced by CD4⁺ T cell lines established from the calves immunized with MSP1aEDₘ alone (Fig. 6). Short-term cultures derived from the negative control calves did not secrete significant amounts of IFN-γ (Fig. 6). The negative control Ags, membranes prepared from uninfected bovine erythrocytes (10⁵ per ml), were added to the cultures at the start of the expansion phase. IFN-γ production was detected by ELISA using a commercial IFN-γ ELISA kit from R&D systems.

Table III. Response of CD4⁺ T lymphocytes to defined MSP1a epitopes

<table>
<thead>
<tr>
<th>Peptide a</th>
<th>Flt3L/GM-CSF + MSP1aEDₘ</th>
<th>VR-1055 + MSP1aEDₘ</th>
<th>Flt3L/GM-CSF + VR-1055</th>
</tr>
</thead>
<tbody>
<tr>
<td>B0019</td>
<td>0.7 (16/22)</td>
<td>0.8</td>
<td>0.5 (24/24)</td>
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<tr>
<td>B0021</td>
<td>3.1</td>
<td>1.8</td>
<td>1.4</td>
</tr>
<tr>
<td>B0020</td>
<td>26.0</td>
<td>26.0</td>
<td>26.0</td>
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<tr>
<td>B0023</td>
<td>29.0</td>
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<tr>
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CD4⁺ T cell-enriched lines were tested in a 72-h proliferation assay with MSP1a peptides. Results are presented as SI. SI > 3.5 are indicated in bold.

Discussion

DNA-encoding Flt3L and GM-CSF significantly increased DC recruitment to the immunization site. Interestingly, the peak recruitment occurred starting at 10 days postinoculation, similar to the peak recruitment at 9–14 days observed when recombinant soluble human Flt3L was inoculated daily into mice (10 µg/day) or humans (10 µg/kg/day) (15, 16). This similarity in peak recruitment between daily administration of soluble Flt3L and a single inoculation of DNA vector-encoded Flt3L and GM-CSF likely reflects the kinetics of DC expansion and trafficking following growth factor stimulation. Notably, the results demonstrate that growth factor delivery via a DNA vaccine vector provided the levels of growth factors sufficient for enhanced DC recruitment. Combining administration of soluble rFlt3L and GM-CSF to mice has been shown to have an additive effect on splenic DC recruitment (20). As experiments dissecting the ability of DNA encoding each hemopoietic growth factor, Flt3L and GM-CSF, vs the combination to recruit DC has not been reported for any species, the experimental system described in this study can be used to determine whether the effects are additive, or synergistic, at the expression levels associated with a single DNA inoculation.
Most importantly, the administration of DNA-encoded Flt3L and GM-CSF before MSP1a DNA immunization resulted in a significant increase in the effector/memory lymphocyte population and significant enhancement of CD4+ T lymphocyte proliferative responses upon restimulation with MSP1a. Priming and expansion of the CD4+ T cell population is the primary goal for an effective A. marginale vaccine (29, 39). Critically, the MSP1a DNA vaccine primed T cells are capable of recognizing A. marginale organisms and native MSP1a, as well as the DNA vector-expressed immunogen, indicating that such effector/memory T cells would be stimulated in vivo following A. marginale challenge.

The enhancement of Ag-specific T cell responses following administration of DNA vector-encoded Flt3L and GM-CSF in calves is comparable to the efficacy reported for inbred mouse strains. Administration of soluble rFlt3L to BALB/c and C57BL/6 mice for 9 days followed by immunization with chicken OVA enhances clonal expansion and proliferation of Ag-specific T cells in vivo and in vitro (21). Similarly, coadministration (9, 44) or coexpression (8, 45) of GM-CSF with Ag significantly enhances specific CD4+ T cell responses in mice. Notably, the enhanced T cell proliferative responses were accompanied by significantly higher levels of IFN-γ when restimulated in vitro (21). In the present study, the dominant proliferative response by MSP1a-specific CD4+ T cell lines established from calves inoculated with DNA-encoding Flt3L and GM-CSF before MSP1a immunization was mirrored by significantly greater (p < 0.05) MSP1a-specific IFN-γ levels. IFN-γ expression is associated with protection in A. marginale vaccinates, most likely functioning through coordinated activation of macrophages and induction of IgG2, the most efficient opsonizing IgG subclass in cattle (31, 39, 46–49). Consistent with this function, the calves treated with Flt3L and GM-CSF before MSP1aEDm immunization developed MSP1a-specific IgG2 and had higher IgG2 titers than control calves immunized with MSP1aEDm alone (data not shown).

Immunization using native MSP1 in adjuvant has been shown to induce protection against virulent challenge (50, 51). The predominant CD4+ T cell response in calves immunized with native MSP1 is directed against epitopes in the region between aa 151–366 and specific conserved epitopes in this region are represented by 11 overlapping peptides (52). Analysis of CD4+ T cell lines established from calves immunized using DNA-encoded MSP1a demonstrated that, with a single exception (peptide F3-1), administration of DNA-encoded Flt3L and GM-CSF before MSP1a immunization resulted in dramatically enhanced proliferation upon peptide stimulation ex vivo (Table III). The strong response to defined epitopes suggests that the DNA vaccine strategy does mimic, at least in part, the epitope specificity of the response induced by native MSP1 immunization. Significantly, analysis of CD4+ T cell clones has previously established that the recognition of peptide F2-5 is MHC class II DR-restricted (52). The identity of the MHC class II DR haplotypes between MSP1a DNA vaccinates in both groups (inoculated with Vical 1055-expressing Flt3L and GM-CSF vs inoculated with Vical 1055 alone before immunization) indicates that the enhanced responses are attributable to the growth factor treatment and are not reflections of differences in ability of specific MHC class II molecules to present Ag.

In summary, we have demonstrated that, as hypothesized, DNA-encoding Flt3L and GM-CSF increases DC recruitment to the immunization site and enhances CD4+ T cell responses to MSP1a expressed in a DNA vaccine vector. Although this is the first report testing the efficacy of coadministered DNA-encoded Flt3L and GM-CSF in any species, the primary significance is in documenting the effect on T cell responses in a species that represents one of the actual target populations to be protected by DNA-based immunization. Specifically for A. marginale vaccine development, the results allow progression to testing whether the DNA-encoded Flt3L and GM-CSF augments protection against virulent challenge in MSP1a vaccinates. In addition, the >50-fold increase in Ag expression resulting from modification of MSP1a to MSP1aEDm without loss of critical T cell epitopes, defines the msp1α gene construct to be tested in DNA vaccine trials. More broadly, the ability of DNA-encoded Flt3L and GM-CSF to enhance DC recruitment and Ag-specific CD4+ T cell responses provides an

![FIGURE 6. Flt3L and GM-CSF enhance IFN-γ production by MSP1a-specific CD4+ T cell lines. Short-term MSP1a-specific CD4+ T cell lines were stimulated for 72 h using 10 µg/ml A. marginale FL homogenate or a 1/50 dilution of COS-7L-expressed MSP1aEDm Ag. Negative controls included 10 µg/ml URBC and a 1/50 dilution of control supernatant from COS-7L cells transfected with VR-1055 vector. Supernatants were tested for IFN-γ production by ELISA and results were presented as units per milliliter. The significance of the difference in IFN-γ production was analyzed by ANOVA followed by multiple pairwise comparison tests with Bonferroni correction. * Statistically significant differences (p < 0.05) between the calves treated with Flt3L/ GM-CSF before MSP1aEDm immunization and both the calves immunized with MSP1aEDm (without Flt3L/GM-CSF treatment) and the calves inoculated with Flt3L/GM-CSF but without MSP1aEDm immunization. ** Statistically significant differences (p < 0.05) between the calves immunized with MSP1aEDm (without Flt3L/GM-CSF treatment) and the calves inoculated with Flt3L/GM-CSF but without MSP1aEDm immunization.](http://www.jimmunol.org/Downloadedfrom)
opportunity to identify additive or synergistic effects of the two hematopoietic growth factors on specific events in Ag uptake, processing, and presentation in outbred animals.

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References


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