# 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

# 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[1]

**Waithaka Mwangi,[2]* Wendy C. Brown,* Harris A. Lewin,† Chris J. Howard,‡ Jayne C. Hope,‡ Timothy V. Baszler,* Patrick Caplazi,* Jeffrey Abbott,* and Guy H. Palmer***

**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-$\gamma$ 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 hemopoietic growth factor fetal liver tyrosine kinase 3 ligand (Flt3L)[3] 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,

*Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164; †Department of Animal Sciences, University of Illinois, Urbana-Champaign, IL 61801; and ‡Compton Laboratory, Institute of Animal Health, Compton, Newbury, United Kingdom

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[2] Address correspondence and reprint requests to Dr. Waithaka Mwangi, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040. E-mail address: waitham@vetmed.wsu.edu

[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 erythroctye; SI, stimulation index.



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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 vaccinates 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′-ATA*GATATC*ATGTGGCTG CAGAACCTGCTTCTCC-3′) introduced an *Eco*RV restriction site (in italics) at the 5′ end and the reverse primer (5′-TAT**GGATCC**TCACT TCTGGGCTGGTTCCCAGC-3′) introduced a *Bam*HI restriction site (in bold) at the 3′ end. The *Eco*RV-*Bam*HI fragment was ligated into *Eco*RV-*Bam*HI digested VR-1055 eukaryotic expression vector (Vical, San Diego, CA) to generate pVRGM-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 pVCL/MSP1$\alpha$ (11) by digestion with *Pst*I and ligation into the *Pst*I-digested VR-1055. A construct containing the *msp1*$\alpha$ gene in the correct orientation was designated pVRMSP1$\alpha$. 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 hydropathic profile was predicted by using the algorithm of Hoffmann (www.ch.embnet.org/software/TMPRED_form.html) and a potential membrane insertion domain (residues 133–158) was deleted. To direct MSP1aED secretion, the human CD5 secretory signal sequence was added (35). The open-reading frame encoding the MSP1aED (residues 1–366) was PCR-amplified from a recombinant plasmid (pVAr1) containing a genomic DNA fragment of the *A. marginale* (Virginia strain) *msp1*$\alpha$ gene (27). The forward primer (5′-ATA**CTGCAG**ATGTCAGCA GAGTATGTGTTCTACC-3′) introduced a *Pst*I restriction site (in bold) at the 5′ end and the reverse primer (5′-T*GGATCC*TACTGTGTAGTAGTGTGTC CGAAGG-3′) introduced a *Bam*HI restriction site (in italics) at the 3′ end. The CD5 signal sequence was PCR-amplified and the product was subcloned into the PCR-Blunt vector (Invitrogen, Carlsbad, CA) to generate pCD5ss. The MSP1$\alpha$ED gene was ligated inframe as a *Pst*I-*Bam*HI fragment into *Pst*I-*Bam*HI-digested pCD5ss to generate pCD5ssMSP1aED. The CD5ssMSP1$\alpha$ED open-reading frame, encoding the modified MSP1aED (MSP1aED$_m$), was released as an *Eco*RV-*Bam*HI fragment and was ligated into *Eco*RV-*Bam*HI digested VR-1055 to generate pVRMSP1$\alpha$ED$_m$.

The pVRFlt3L, pVRGM-CSF, pVRMSP1$\alpha$, and pVRMSP1$\alpha$ED$_m$ constructs, as well as the unmodified VR-1055 vector, were amplified in DH5$\alpha$ *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 (BioWhittaker, Walkersville, MD) and was <5 EU/mg in all samples.

### *Protein expression*

Flt3L, GM-CSF, MSP1a, and MSP1aED$_m$ 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 $\mu$g/ml anti-bovine GM-CSF 20.1 mAb (VMRD, Pullman, WA). The pVRMSP1$\alpha$- and pVRMSP1$\alpha$ED$_m$-transfected COS-7L cell monolayers were incubated with 5 $\mu$g/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 microscope was also used to count pVRMSP1$\alpha$- and pVRMSP1$\alpha$ED$_m$-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 4 mM L-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 antiserum 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 ($1 \times 10^4$ cells per well) were incubated in triplicate with dilutions of the GM-CSF supernatant for 7 days at 37°C with 5% $CO_2$ in a humidified chamber. Supernatant from the COS-7L cells transfected with the VR-1055 vector was used as a negative control. The cells were radiolabeled with 0.25 $\mu$Ci/ml [$^3$H]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 [$^3$H]thymidine incorporation (cpm ± 1 SD) was plotted against supernatant dilutions.

### *Induction of MSP1-specific CD4$^+$ T cell recall responses with MSP1aED$_m$ Ag*

Induction of MSP1-specific T cell recall responses by the COS-7L-expressed MSP1aED$_m$ Ag was verified by lymphocyte proliferation assays as described (31). Briefly, CD4$^+$ T cell clones 87.2A1 and 93.4E4 ($3 \times 10^4$ cells per well; Ref. 31) generated from calves immunized with native MSP1 were incubated in duplicate with serial dilutions of the MSP1aED$_m$ COS-7L supernatant and $2 \times 10^5$ irradiated autologous PBMC for 3 days. rMSP1a carboxyl region (0.2–10.0 $\mu$g/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$\mu$Ci/ml [$^3$H]thymidine and harvested as described above. The mean [$^3$H]thymidine incorporation (mean cpm ± 1 SD) was plotted against supernatant dilutions.

### *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 $\mu$l per site) were administered randomly in a defined area (within a radius of 10 centimeters) on the right flank region (immunization site) using a 25-gauge needle. The calves were boosted with 2 mg of the pVRMSP1$\alpha$ED$_m$ construct or the VR-1055 vector at 8-wk intervals.

### *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 $\mu$m) 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 $\mu$g/ml TH97A mAb (Monoclonal Ab Center, Washington State University, Pullman, WA) that recognizes the bovine ortholog 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 a 1/10,000 dilution of streptavidin-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 mm$^2$ 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 $p \leq 0.05$ was considered significant.

### *Establishment of T lymphocyte lines*

Short-term *A. marginale*-specific T lymphocyte lines were established several times from PBMC of the DNA-immunized calves 13-mo postimmunization as described previously (11, 31). Briefly, CD8$^+$ and $\gamma\delta$ T lymphocytes were depleted by incubating PBMC with CD8-specific mAb 7C2B and $\gamma\delta$ 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$^-$, $\gamma\delta^-$ PBMC by stimulation with homogenate prepared from the Florida (FL) strain of *A. marginale*. Briefly, $4 \times 10^6$ CD8$^-$, $\gamma\delta^-$ 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 $\mu$g/ml *A. marginale* FL strain homogenate. Background proliferation was lowered by subculturing cells, without Ag, to a density of $7.5 \times 10^5$ cells/well and cultured with $2 \times 10^6$ 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*

Differentiation 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 $\gamma$-chain of the $\gamma\delta$ TCR-specific mAbs used have been described previously (31).

### *Proliferation assays*

Proliferation assays were conducted as described (11, 31). Briefly, T lymphocyte lines ($3 \times 10^4$ cells) were cultured in triplicate wells of round-bottom 96-well plates (Costar) for 3 days in a total volume of 100 $\mu$l of complete medium containing Ag and $2 \times 10^5$ irradiated autologous PBMC as APC. Ags consisted of *A. marginale* FL strain homogenate (0.4 to 10.0 $\mu$g/ml), rMSP1a carboxyl region (residues 242–540) expressed as a maltose-binding protein (MBP) fusion protein (0.4 to10.0 $\mu$g/ml) (31), and the COS-7L-expressed MSP1aED$_m$ supernatant (1/10–1/6250 dilution). Peptides F2.1-F2.5 and F3.1-F3.6 (0.4–10.0 $\mu$g/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) regions of the extracellular domain of MSP1a (27). These regions were defined by dividing residues 242–540 into three fragments (F1-F3) of ~112 residues. COS-7L-expressed F2 and F3 proteins induced MSP1a-specific recall responses and thus, F2 and F3 peptides were generated to map T cell epitopes within these regions (52). Membranes prepared from uninfected bovine erythrocytes (URBC), rMBP (New England Biolabs, Beverly, MA), the supernatant from 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 $p \leq 0.05$ was considered significant.

### *Detection of IFN-$\gamma$ in supernatants of T lymphocyte lines*

Short-term *A. marginale*-specific T lymphocyte lines were established as above and then were restimulated for 72 h with autologous APC and *A. marginale* FL strain homogenate (10 $\mu$g/ml), rMSP1a carboxyl region-MBP fusion protein (10 $\mu$g/ml), and the MSP1aED COS-7L supernatant (1/50 dilution); supernatants were tested for IFN-$\gamma$ production by ELISA. Controls consisted of supernatants from cell lines stimulated with membranes prepared from uninfected bovine erythrocytes (10 $\mu$g/ml), rMBP (10 $\mu$g/ml), and the VR-1055 COS-7L supernatant (1/50 dilution). The bovine IFN-$\gamma$ assay was performed using an ELISA kit (BOVIGAM; CSL, Parkville, Victoria, Australia) according to the manufacturer's protocol. The IFN-$\gamma$ activity in culture supernatants diluted 1/4–1/100 was determined by comparison with a standard curve obtained as described previously (39). The results are presented as units of IFN-$\gamma$ per milliliter. The significance of the differences in units of IFN-$\gamma$ per milliliter secreted by T

Table I. *DNA immunization protocol*

| Group | Flt3L/GM-CSF + MSP1aED$_m$ | VR-1055 + MSP1aED$_m$ | Flt3L/GM-CSF + VR-1055 |
|---|---|---|---|
| Animal numbers | B0019; B0021 | B0020; B0023 | B0018; B0024 |
| DRB3[a] | 16/22; 16/24 | 16/22; 16/24 | 24/24; 22/24 |
| Day 1 treatment[b] | Flt3L + GM-CSF | VR-1055 | Flt3L + GM-CSF |
| Day 9 treatment[c] | MSP1aED$_m$ | MSP1aED$_m$ | VR-1055 |

[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 Flt3L 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.

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 MSP1a-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 (CC302) 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 ($5 \times 10^5$ or $1 \times 10^6$ cells/well) were added to each well in 50-μl volumes. Ags or control mitogens were then added in 50-μl volumes. MSP1aED$_m$ was tested using supernatants of pVRMSP1αED$_m$-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

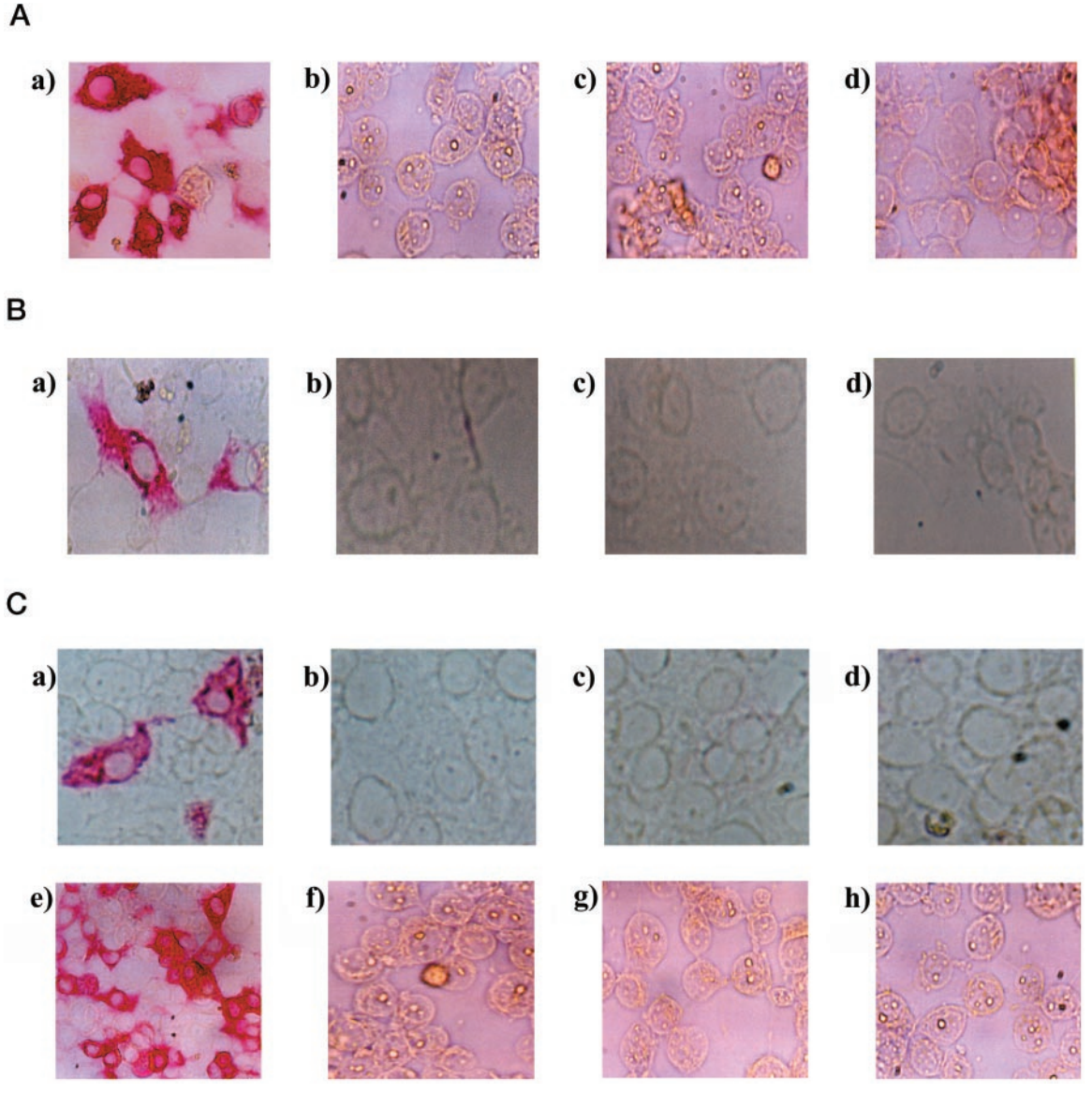


**FIGURE 1.** *A*, Expression of bovine Flt3L in COS7L cells. Monolayers of COS-7L cell transfectants were assayed for Flt3L expression using a murine anti-bovine Flt3L peptide serum by in situ immunocytochemistry. *Aa* and *Ab*, COS-7L cells transfected with the pVRFlt3L construct encoding bovine Flt3L; *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* MSP1a and MSP1aED$_m$ in COS-7L cells. COS-7L transfectants were assayed for MSP1a and MSP1aED$_m$ expression using Ana22B1 mAb by in situ immunocytochemistry. *Ca* and *Cb*, COS-7L cells transfected with the pVRMSP1α construct encoding full-length *A. marginale* MSP1a (Virginia strain); *Ce* and *Cf*, COS-7L cells transfected with the pVRMSP1αED$_m$ construct encoding MSP1aED$_m$; *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.

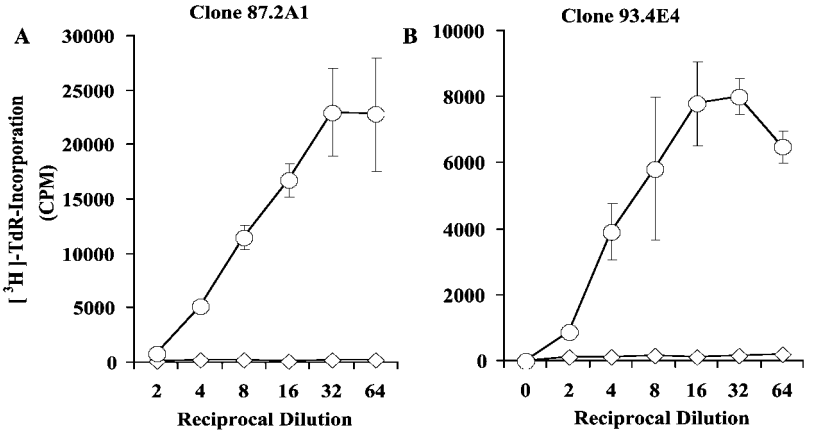


**FIGURE 2.** COS-7L expressed MSP1aED$_m$ Ag induces a CD4$^+$ T cell recall response by T cells from native MSP1-immunized calves. Induction of MSP1-specific T cell recall responses by the COS-7L-expressed MSP1aED$_m$ Ag was verified by lymphocyte proliferation assays using MSP1-specific CD4$^+$ T cell clones. *A*, clone 87.2A1; *B*, clone 93.4E4. ○, COS-7L-expressed MSP1aED$_m$ Ag; ◇, supernatant from COS-7L cells transfected with the VR-1055 vector. Each point represents the mean of [$^3$H]thymidine incorporation in cpm from three wells ± SD.

(42). The negative controls were supernatants of COS-7L cells at a dilution of 1/40 and complete RPMI alone. The plates were wrapped loosely with aluminum foil (43) and incubated for 36 h at 37°C with 5% $CO_2$. Plates were then washed six times with PBST, once with distilled water, six times with PBST, and then twice using PBS. Biotinylated mouse anti-bovine IFN-γ mAb CC302 (0.5 μg/ml) diluted in 1% BSA/PBS, was added and the plates were incubated for 2 h at room temperature. Excess Ab was removed by washing using PBST. Vectastain ABC peroxidase PK-4000 kit (Vector Laboratories, Burlingame, CA) was mixed and 100 μl per well added. After a 1.5-h incubation at room temperature, plates were washed six times with PBST followed by two washes using PBS. The spots were developed with AEC (Sigma-Aldrich) according to the manufacturer's recommendations. Plates were dried overnight and read using an ELISPOT reader and AID 2.9 software (AutoImmun Diagnostika, Strassberg, Germany). For each animal, the mean of spots in negative control wells was subtracted from the mean of spots in test wells to determine the number of MSP1a-specific spot-forming cells. Results were presented as the number of MSP1a-specific IFN-γ secreting cells per $10^6$ PBMC (±SD) and the data was analyzed by ANOVA with Bonferoni correction to identify statistical differences in treatment groups.

## 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 assessed 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 Flt3L has been reported previously (33). 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 MSP1αED$_m$*

Immunocytochemical detection using mAb Ana22B1 revealed expression in COS-7L cells transfected with either pVRMSP1α or pVRMSP1αED$_m$ (Fig. 1*C*). 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 pVRMSP1αED$_m$ transfectants consistently had an expression efficiency >50% (Fig. 1*C*; data not shown). Dot blot analysis of concentrated supernatants generated from the COS-7L cell transfectants showed that MSP1αED$_m$ 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 MSP1aED$_m$ 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 MSP1a (clone 87.2A1, maximal SI = 50.7, and clone 93.4E4, maximal SI = 22.2). This result shows that the MSP1aED$_m$ Ag stimulates CD4$^+$ T cells primed using native MSP1, and therefore bears epitopes represented on the native protein. Consequently, the pVRMSP1αED$_m$ 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

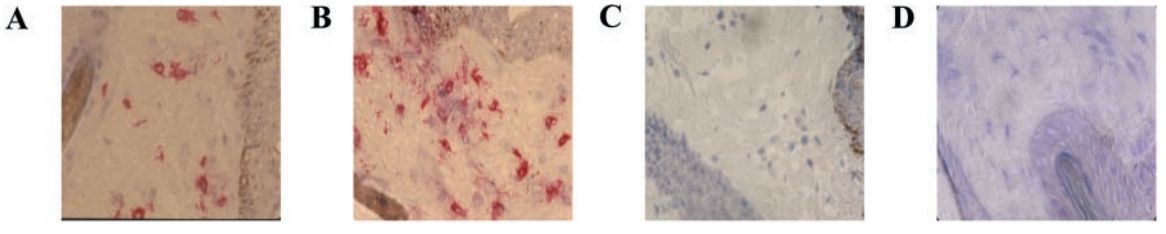


**FIGURE 3.** Flt3L and GM-CSF enhance DC recruitment to the skin. Recruitment of DC to the skin was analyzed by quantitative immunohistochemistry using anti-CD1 mAb TH97A. *A*, Cryostat section from a preinoculation biopsy probed with mAb TH97A; *B*, cryostat section from a day 10 biopsy probed with 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.

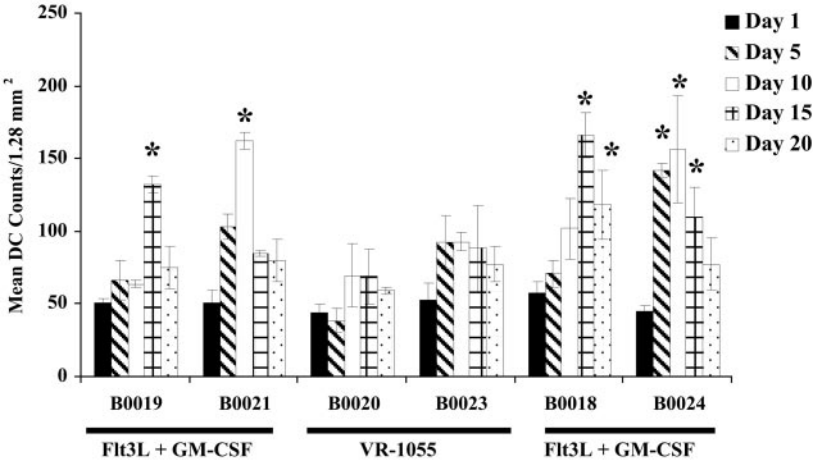


**FIGURE 4.** Kinetics of skin DC recruitment following Flt3L and GM-CSF DNA inoculation. Holstein calves were inoculated intradermally with a mixture of pVRFlt3L and pVRGM-CSF (1 mg each) or with pVR-1055 vector (2 mg). Data are presented as the mean ± SD of DC per 1.28 $mm^2$ using anti-CD1 mAb TH97A staining. The significance of the difference between means of the treated and the control groups, and the variation of the means over time was analyzed by ANOVA followed by multiple pairwise comparison tests with Bonferroni correction. *, Statistically significant differences ($p < 0.05$) between the Flt3L/GM-CSF-treated calves and the vector control-treated calves as well as between the postinoculation timepoint and the preinoculation biopsies in the Flt3L/GM-CSF treated calves.

$CD1^+$ 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 injected with the VR-1055 vector alone.

### *Flt3L and GM-CSF enhance Ag-specific CD4+ T cell responses*

The ability of Flt3L and GM-CSF to enhance MSP1a-specific $CD4^+$ T cell responses was assessed by proliferation assays using short-term T cell lines depleted of $CD8^+$ and $\gamma\delta$ $TCR^+$ T lymphocytes. Cell surface phenotypic analysis by flow cytometry showed that the T cell lines comprised >86% $CD4^+$ T cells, and <1% CD8 and $\gamma\delta$ T cells (data not shown). The $CD4^+$ T cell lines established from the Flt3L/GM-CSF-treated and $MSP1aED_m$-immunized calves had significantly higher proliferative responses (SI > 30) to both *A. marginale* FL Ag (10 $\mu$g/ml) and the COS-7L-expressed MSP1aEDm (1/50 dilution) supernatant compared with proliferative responses (SI < 15) to the same Ags by the $CD4^+$ T cell lines established from the calves immunized with $MSP1aED_m$ alone (Fig. 5). A similar difference in proliferative responses to rMSP1a carboxyl region (10.0 $\mu$g/ml) as well as to lower concentrations of all the Ags tested was observed (data not shown). All $CD4^+$ T cell lines established from calves immunized with the $MSP1aED_m$ DNA construct, but not those established from control calves, proliferated in a dose-dependent manner in response to *A. marginale* FL strain homogenate (0.4 to 10.0 $\mu$g/ml), rMSP1a carboxyl region (aa 242–767) expressed as an MBP fusion protein (0.4–10.0 $\mu$g/ml) (31), and the COS-7L-expressed $MSP1aED_m$ supernatant (1/10–1/6250 dilution). There was no response to membranes prepared from URBC, rMBP, and the supernatant from COS-7L cells transfected with the VR-1055 vector (data not shown). The MSP1a-specific proliferative responses of $CD4^+$ T cell lines from the $MSP1aED_m$-immunized calves pretreated with Flt3L/GM-CSF were significantly higher than those of $MSP1aED_m$-immunized calves that did not receive Flt3L/GM-CSF ($p < 0.05$). The responses of $CD4^+$ T cell lines from the $MSP1aED_m$-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-$\gamma$ ELISPOT assay using both $5 \times 10^5$ and $1 \times 10^6$ PBMC per well. The number of MSP1a-specific cells in $MSP1aED_m$-immunized calves pretreated with Flt3L/GM-CSF was significantly higher ($p < 0.05$) than those of $MSP1aED_m$-immunized calves that did not receive cytokines (Table II). Pairwise comparison between animals with identical DR haplotypes in these two groups, B0019 with B0020 (both are 16/22) and B0021 with B0023 (both are 16/24), also demonstrated significantly higher ($p < 0.05$) numbers of MSP1a-specific effector/memory cells in the $MSP1aED_m$-immunized calves pretreated with Flt3L/GM-CSF (Table II).

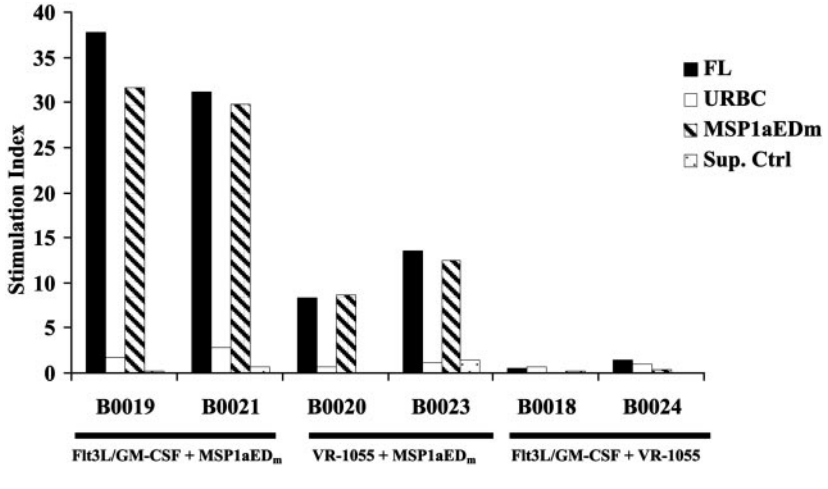


**FIGURE 5.** DNA-encoded Flt3L and GM-CSF enhance $CD4^+$ T cell responses to MSP1a. $CD4^+$ T cell lines established from each vaccinate were stimulated for 72 h using 10 $\mu$g/ml *A. marginale* FL strain homogenate (■) and a 1/50 dilution of COS-7L-expressed $MSP1aED_m$ Ag (▧). Negative controls included 10 $\mu$g/ml URBC membranes (□) and a 1/50 dilution of control supernatant (Sup. Ctrl) from COS-7L cells transfected with VR-1055 vector (▨). Results are presented as the SI. The significance of the differences in $CD4^+$ T cell line response (counts per minute) to all the Ags tested was analyzed by ANOVA followed by multiple pairwise comparison tests with Bonferroni correction and using $p \leq 0.05$. This assay was repeated three times with similar results.

Table II. *Enumeration of MSP1a-specific lymphocytes in PBMC of vaccinated calves*

| Group | Animal | Average Number of IFN-γ Secreting Cells Per $10^6$ PBMC (±SD) | |
|---|---|---|---|
| | | $5 \times 10^5$ PBMC/well | $1 \times 10^6$ PBMC/well |
| Flt3L/GM-CSF + $MSP1aED_m$ | B0019 | 359 ± 34[a] | 264 ± 7[a] |
| | B0021 | 441 ± 17[a] | 284 ± 19[a] |
| $MSP1aED_m$ | B0020 | 40 ± 14 | 15 ± 6 |
| | B0023 | 223 ± 90 | 183 ± 7 |
| Flt3L/GM-CSF | B0018 | 4 ± 17 | 12 ± 9 |
| | B0024 | 14 ± 25 | 27 ± 7 |

[a] Significantly greater ($p < 0.05$) than B0020, B0023, B0018, and B0024.

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 MSP1 (52). The $CD4^+$ T cell lines established from calves treated with Flt3L/GM-CSF before $MSP1aED_m$ immunization had significantly higher proliferative responses to the MSP1a peptides than $CD4^+$ T cell lines established from the calves immunized with $MSP1aED_m$ alone (Table III). These data confirmed that the $CD4^+$ T cells induced by $MSP1aED_m$ 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 $MSP1aED_m$-immunized calves compared with that produced by $CD4^+$ T cell lines established from the calves immunized with $MSP1aED_m$ 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 (10 μg/ml), and the supernatant from COS-7L cells transfected with the VR-1055 vector (1/50 dilution) stimulated undetectable or minimal levels of IFN-γ production from all cultures (Fig. 6).

## 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.

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

| Peptide[b] | Proliferative Response[a] (SI) of $CD4^+$ T Cell Lines | | | | | |
|---|---|---|---|---|---|---|
| | Flt3L/GM-CSF + $MSP1aED_m$ | | VR-1055 + $MSP1aED_m$ | | Flt3L/GM-CSF + VR-1055 | |
| | B0019 (16/22)[c] | B0021 (16/24) | B0020 (16/22) | B0023 (16/24) | B0018 (24/24) | B0024 (22/24) |
| MSP2 P1 | 0.7 | 3.1 | 0.8 | 1.8 | 0.5 | 1.4 |
| F2–1 | **29.0** | **26.0** | **4.9** | **9.4** | 0.5 | 1.5 |
| F2–2 | **43.8** | **26.2** | **8.7** | **7.8** | 1.2 | 1.9 |
| F2–3 | **25.1** | **23.9** | 2.8 | **8.5** | 1.1 | 1.4 |
| F2–4 | **30.2** | **29.0** | **7.0** | **6.9** | 1.8 | 2.5 |
| F2–5 | **30.3** | **29.8** | 1.8 | **5.9** | 1.8 | 1.6 |
| F3–1 | 1.7 | **12.1** | **4.1** | **3.6** | 1.1 | 2.3 |
| F3–2 | **22.3** | **24.9** | **6.5** | **3.9** | 0.9 | 2.4 |
| F3–3 | **26.1** | **26.9** | **6.0** | **5.8** | 1.9 | 1.2 |
| F3–4 | **17.9** | **16.4** | **4.1** | **5.9** | 1.8 | 1.3 |
| F3–5 | **33.9** | **28.8** | 1.3 | **11.7** | 1.0 | 1.8 |
| F3–6 | 2.7 | **14.2** | 1.9 | 1.6 | 1.0 | 1.2 |

[a] $CD4^+$ 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.

[b] Peptides F2–1 to F2–5 and F3–1 to F3–6 represent MSP1a T cell epitopes as described and referenced in *Materials and Methods*. The MSP2 P1 peptide is from an unrelated *A. marginale* protein and was used as a negative control.

[c] Bovine MHC class II-*DRB3* alleles were defined by PCR-restriction fragment length polymorphism analysis of exon 2 (36).

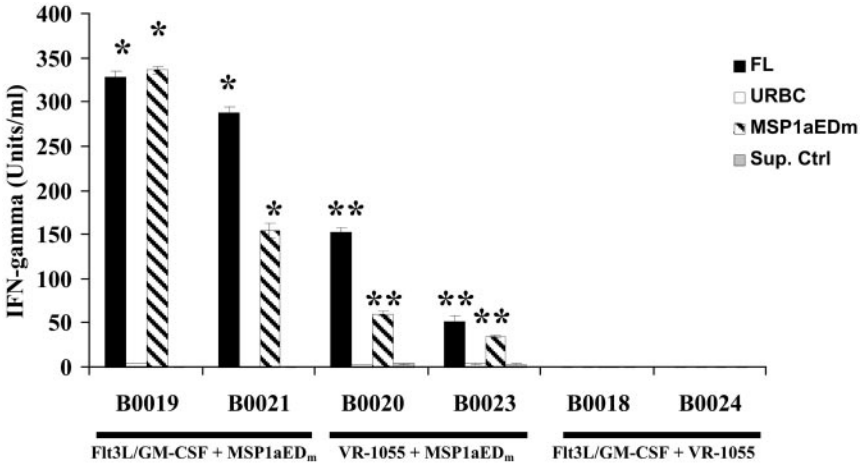


**FIGURE 6.** Flt3L and GM-CSF enhance IFN-γ production by MSP1a-specific CD4+ T cell lines. Short-term MSP1a-specific CD4+ T cell lines were stimulated for 72 h using 10 μg/ml *A. marginale* FL homogenate or a 1/50 dilution of COS-7L-expressed MSP1aED$_m$ Ag. Negative controls included 10 μg/ml URBC 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 MSP1aED$_m$ immunization and both the calves immunized with MSP1aED$_m$ (without Flt3L/GM-CSF treatment) and the calves inoculated with Flt3L/GM-CSF but without MSP1aED$_m$ immunization. **, Statistically significant differences ($p < 0.05$) between the calves immunized with MSP1aED$_m$ (without Flt3L/GM-CSF treatment) and the calves inoculated with Flt3L/GM-CSF but without MSP1aED$_m$ immunization.

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 MSP1aED$_m$ immunization developed MSP1a-specific IgG2 and had higher IgG2 titers than control calves immunized with MSP1aED$_m$ 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 MSP1aED$_m$, 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

opportunity to identify additive or synergistic effects of the two hemopoietic growth factors on specific events in Ag uptake, processing, and presentation in outbred animals.

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