Characterization of a Phenotypically Unique Population of CD13+ Dendritic Cells Resident in the Spleen

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Immature dendritic cells (DCs) resident in bovine spleens represent a distinct CD11a+ CD11c+ CD13+ CD172a+ CD205+ population compared to those circulating in peripheral blood or trafficking via afferent lymph. Upon cytokine-induced maturation, splenic DCs both efficiently present antigen in the stimulation of allogeneic lymphocyte proliferation and recall antigen-specific responses.

Dendritic cells (DCs) initiate adaptive immune responses by taking up an antigen and, following their maturation and migration to T-lymphocyte-rich zones, presenting the antigen to T cells in the context of the major histocompatibility complex (MHC) and costimulatory molecules (2, 31). Specific vaccine targeting of DCs has been shown to enhance priming and to convert nonimmunogenic tumor antigens into effective immunogens that induce protective immunity (3). Using DNA vaccine vectors expressing the Flt3 ligand and granulocyte-macrophage colony-stimulating factor (GM-CSF), along with a microbial antigen, we have shown that increased recruitment of DCs to the skin enhances the efficacy of DNA vaccines in cattle and that the CD4+ T-lymphocyte responses are significantly amplified (23). However, there is strong evidence that priming, expansion, and maintenance of memory T lymphocytes and trafficking of these T cells upon challenge can be organ specific and are greatly influenced by the site of initial antigen presentation (6, 17, 21). Importantly, DC lineages differ among organs, and thus contemporary approaches that modify vaccine vectors to enhance the transfection and expression of vaccine antigens in organ-specific DCs may require different targeting strategies.

While DC lineages have been best studied in mice and humans, there is clear evidence for different lineages in cattle with functional differences in their abilities to stimulate CD4+ and CD8+ T cell responses (12, 28). However, these studies have not examined the spleen, the critical organ for priming and expansion of the immune response against blood-borne parasites (4, 8, 26, 27). Consistent with the critical role of the spleen in initiating protective immunity, splenectomy markedly delays the development of antigen-specific immune responses following infection with the blood parasites Anaplasma and Babesia spp., resulting in severe disease and, usually, death (16, 18). With a long-term goal of developing novel vaccines that will effectively induce immune responses that control these important hemoparasitic diseases of cattle, we are focused on improving our understanding of how immune responses are initiated and expanded in the spleen. The objective of the present study was to characterize splenic DCs and determine if they are phenotypically distinct from peripheral blood DCs and previously described bovine DC lineages obtained from afferent lymph (9, 12, 13).

Spleens were surgically removed from healthy male Holstein calves and, rinsed in phosphate-buffered saline (PBS) containing 20% (vol/vol) acid citrate dextrose (ACD) with 100 U penicillin and 100 μg streptomycin per ml. The spleen was mechanically disrupted using a tissue grinder, and cells were obtained by passing small fragments through a 100-μm-pore-size nylon cell strainer (BD Falcon). Spleen cells were centrifuged at 430 × g and resuspended in four volumes of Tris-buffered 0.87% ammonium chloride for 10 min. Remaining cells were washed in PBS-ACD, suspended in fetal bovine serum containing 10% dimethyl sulfoxide, and cryopreserved in liquid nitrogen. Peripheral blood mononuclear cells (PBMC) were isolated from the same calves and cryopreserved in liquid nitrogen using the same procedure as used for the spleen cells. B lymphocytes and monocytes were isolated from PBMC by positive selection using, respectively, the monoclonal antibodies (MAbs) BAQ44A and CAM66A (Table 1). Following 30 min of incubation at 4°C with the appropriate MAb, the cells were washed three times in PBS, incubated with goat anti-murine immunoglobulin M (IgM) microbeads (Miltenyi Biotec), and positively selected using a magnetic field. Macrophages were derived by culture of adherent PBMC in complete RPMI 1640 medium on 100-mm petri dishes (Becton Dickinson) at 37°C in 5% CO2 for 7 days. Accutase (Innovative Cell Technologies) was used to collect adherent cells.

The strategy used to identify splenic DC populations was to
Table 1. Monoclonal antibodies used for phenotypic analysis and cell sorting

| Antibody | Isotype | Specificity | Source |
|----------|---------|-------------|--------|
| H34A     | IgG2b   | MHC class II | VMRD   |
| TH22A    | IgG2a   | MHC class II | VMRD   |
| BAQ44    | IgM     | B cell      | VMRD   |
| GB25A    | IgG1    | B cell      | VMRD   |
| CAM66A   | IgM     | CD14        | VMRD   |
| CAM36A   | IgG1    | CD14        | VMRD   |
| CACT31A  | IgM     | CD2         | VMRD   |
| BAQ95A   | IgG1    | CD2         | VMRD   |
| MM1A     | IgG1    | CD3         | VMRD   |
| CACT61A  | IgM     | γδ T-cell receptor δ chain | VMRD |
| CACT81A  | IgG1    | γδ T-cell receptor γ chain | VMRD |
| GB21A    | IgG2b   | γδ T-cell receptor δ chain | VMRD |
| IL-11A   | IgG2a   | CD4         | VMRD   |
| CC30     | IgG1    | CD4         | IAH    |
| CACT80C  | IgG1    | CD8         | VMRD   |
| CC13     | IgG1    | CD1         | Serotec |
| HUH73A   | IgG1    | CD11a       | VMRD   |
| MM12A    | IgG1    | CD11b       | VMRD   |
| BAQ153A  | IgG1    | CD11c       | VMRD   |
| DH59B    | IgG1    | CD17a       | VMRD   |
| GC42A    | IgG1    | CD45RO      | IAH    |
| ILA116   | IgG3    | CD45RO      | IAH    |
| CC81     | IgG1    | CD13        | IAH    |
| CC98     | IgG2b   | CD205       | IAH    |
| IL-A159  | IgG1    | CD80        | IAH/EU |
| IL-A190  | IgG1    | CD86        | IAH/EU |
| AKS1     | IgG1    | CD335 (NK cells [30]) | NVS    |

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Sorted putative splenic DCs were then cultured in complete RPMI 1640 medium supplemented with 200 ng/ml of recombinant bovine interleukin 4 (IL-4) (10, 24), 100 ng/ml recombinant bovine GM-CSF (23, 24), 100 ng/ml recombinant bovine Flt3 ligand (22, 24), and 10 μg/ml recombinant bovine CD40 ligand (rbCD40L). To obtain bovine CD40L, a DNA construct encoding the extracellular domain of bovine CD40L (CD40L-ED) linked in-frame with the sequence encoding the CD5 secretory signal sequence (7) was generated in the expression vector VR-1055 (Vical). A bovine CD40L-ED-specific forward primer (5′ ATTACTGCAGATGTTATCTTTCAAGAGATTG 3′) and a bovine CD40L reverse primer (5′ ATAGGATTTCACTTTATCGTCATCGTCCTTGTAGTC 3′) were used to PCR amplify the CD40L-ED open reading frame from cDNA generated from bovine T lymphocytes stimulated with ionomycin and phorbol myristic acetate as previously described (11). The reverse primer was extended to include complementary sequence (in bold) of the codons encoding the FLAG tag (amino acid sequence, DYKDDDDK) (20) and also introduced a BamHI restriction site (in italics) at...
FIG. 3. Morphology of bovine splenic DCs. Immature DCs immediately after sorting (A) and mature DCs after 72 h of culture at magnifications of ×300 (B) and ×600 (C) are shown.

FIG. 4. Comparison of the abilities of different antigen-presenting cells to stimulate an allogeneic lymphocyte response and correlation with activation phenotype. (A) Stimulation of allogeneic lymphocyte proliferation by increasing numbers of mature splenic or peripheral blood DCs, macrophages, B lymphocytes, or monocytes. Means ± standard deviations of results from triplicate wells are shown. The response induced by monocytes was identical to that of B lymphocytes and thus is not shown. (B) MHC class II, CD80, and CD86 surface molecule expression on mature splenic and peripheral blood DCs and macrophages (black profile). The background of secondary antibody binding is indicated by the gray profile. TdR, thymidine.
the 3’ end of the PCR product, designated cd40l\(_{\text{flag}}\). The
CD5 secretory signal sequence was added at the 5’ end of
cd40l\(_{\text{flag}}\) by PCR using two overlapping primers (5’ ACCT
TGATACCCCTGGGATGCTGTGCTTCCTGCTCTGC
GACTGACATGTTGCTATCCTACAGACG 3’ and 5’ AT
AGATATCCACATGCCATGGGTCTCTGCAACC
GGCCACACCTTGACTGTGGGATGCTGT 3’), and the second primer introduced an EcoRV restriction site (in bold)
at the 5’ end of the PCR product. The resultant construct,
designated cd5cd40l\(_{\text{flag}}\), was EcoRV-BamHI digested and
subcloned into the VR-1055 eukaryotic expression vector to
generate a construct designated CD40L-ED. rbCD40L was
expressed as FLAG-tagged protein in 293 Free-Style cells
(Invitrogen) and affinity purified using Anti-FLAG M2-agarose
gel (Sigma) as previously described (24). Purified protein
was then tested for biological activity with B lymphocytes
positively selected with magnetic beads from PBMC using a
modified protocol of a previous study (29). Briefly, B
lymphocytes (2 \times 10^5 cells/well) were incubated in triplicate
with 200 ng/ml recombinant bovine IL-4 (10, 24) either
alone or in combination with rbCD40L in a concentration
range between 0.1 ng to 10 \mu g per ml. Cells were incubated
for 72 h at 37°C with 5% CO\(_2\), and proliferation was
measured by radiolabeling with 0.25 \mu Ci of [\(^3\)H]thymidine over
the last 18 h of culture. Cells were collected using an
automated cell harvester (Tomtec), and incorporated [\(^3\)H]thymidine was counted with a liquid scintillation counter.
The stimulation of significant dose-dependent proliferation of
B lymphocytes (1, 19) demonstrated that the recombinant
cytokine was biologically active (Fig. 2).

Microscopic examination of the putative splenic DCs imme-
diately after sorting revealed a rounded appearance (Fig. 3A).
Small dendrites appeared following overnight culture, and
numerous long cytoplasmic veils and aggregates, typical of
fully mature DCs, developed within 72 h (Fig. 3B and C). To ex-
amine the ability of these mature DCs to present antigen
and stimulate T cells, two assays were performed. The first was
an assay for the ability of these mature DCs to present antigen
and mature DCs, as described above. DCs sorted from the
spleen and from peripheral blood induced significantly higher
(P < 0.05) proliferative responses of allogeneic lymphocytes
than did purified macrophages, monocytes, or B lymphocytes
(Fig. 4A). The magnitude of lymphocyte proliferation was propor-
tional to the number of DCs added (Fig. 4A) and the comparative
effectiveness of the DCs as antigen-presenting cells associ-
ated with the surface expression of MHC class II, CD80, and
CD86 molecules (Fig. 4B).

The second functional assay measured the ability of DCs to
stimulate antigen-specific recall responses. A short-term T-
lymphocyte cell line was established from a calf (MHC class II
DRB3 *1201/*1201) immunized with Anaplasma marginale
MSP2 protein (5). Briefly, CD8- and \(\gamma\delta\) T-cell-depleted PBMC
were stimulated with MSP2 for 2 weeks, followed by a 1-week
rest as previously described (23). The specific MSP2 peptide
P16-7, previously shown to be presented by the MHC class II
DRB3 *1201 allele (25), or a negative control peptide, P1,
were added to autologous splenic DCs or macrophages as antigen-presenting cells and cultured with 3 \times 10^4 cells of the
T-lymphocyte cell line. After 72 h, cells were pulsed with
[\(^3\)H]thymidine and harvested as described above. Both splenic
DCs and macrophages presented antigen and induced antigen-
specific CD4^\(+\) T-cell recall responses, with significantly higher
responses (P < 0.05) stimulated by DCs when either 300 or
3,000 antigen-presenting cells were used (Fig. 5).

To identify the surface phenotype of these splenic DCs,
monoclonal antibodies recognizing a series of both activation
and surface markers previously identified on bovine
afferent lymph DCs were used in multicolor flow cytometry.
DCs (MHCII^+ CD2^+ B cell^- \(\gamma\delta\) CD14^-) derived from either
peripheral blood or spleen were incubated with MAb specific
each of the following cell surface markers: CD1, CD11a,
CD11b, CD11c, CD13, CD45RO, CD80, CD86, CD172a, and
CD205 (Table 1). After incubation for 30 min on ice, cells were
washed three times in complete RPMI 1640 medium containing
0.02% sodium azide by centrifugation at 300 \times g. Fluores-
cecin isothiocyanate-, PE-, allophycocyanin-, or PE-Cy5-conju-
gated isotype-specific goat anti-mouse antibodies were used as
secondary antibodies (Caltag Laboratories and Southern Bio-
tech). After incubation for 15 min on ice, labeled cells were
washed twice in cold PBS and fixed in 1% formaldehyde.
A minimum of 80,000 labeled cells were analyzed by flow cytom-
etry using a FACSort (Becton Dickinson). Splenic and peripheral
blood DCs were clearly distinct from the B lymphocytes,
monocytes, and macrophages, being negative for these lineage-
specific markers (data not shown). The splenic DCs were phe-
notypically distinct from both the peripheral blood DCs and
the two previously described types of afferent lymph DCs (Ta-
ble 2). Between DCs derived from peripheral blood versus
spleen, the major surface phenotypic difference was the high
level of CD13 (14) expression on the splenic DCs (Fig. 6). The
expression of this marker, which is a type II transmembrane
protein, was not linked to differential activation status, as both sets of DCs revealed very low or no expression of CD80 or CD86 (Table 2). Splenic DCs also displayed expression levels of CD11b, CD11c, and CD205 different from those of peripheral blood DCs (Fig. 6). CD205, a type I cell surface glycoprotein, is expressed on different DC lineages in mice (15) and has been used as a lineage marker to isolate DCs from the large-sized bovine afferent lymph veiled cells (9, 12, 13). Examination of total gated CD205+ cells in the bovine spleen included both B (CD21+) and T lymphocytes (CD3+) (data not shown). Thus, although the splenic DCs uniformly expressed CD205, CD205 cannot be used as a specific DC marker or as a specific targeting molecule for vaccine delivery. Splenic DCs were also distinct from the two well-described subsets of afferent lymph DCs (12, 13) based on multiple discriminatory surface markers (Table 2). Splenic DCs did not express CD45RO or CD1, which are expressed on both subsets of afferent lymph DCs. Uncultured splenic DCs also exhibited an immature phenotype, as indicated by the low level of CD80/86 expression, while the higher level of expression on the afferent lymph DCs is indicative of a more mature phenotype.

Maturation of splenic DCs using IL-4, the Flt3 ligand, GM-CSF, and CD40L induced strong expression of both CD80 and CD86 (Fig. 4B) concomitantly with a complete loss of CD13 (data not shown). This mature phenotype was linked to the capacity of the DCs to present antigen effectively (Fig. 4A and B). Comparison with identically cultured peripheral blood DCs revealed differences in expression of all three CD11 molecules: mature splenic DCs had higher levels of CD11a and CD11c expression but lower levels of CD11b than mature peripheral blood DCs. Thus, the two populations of DCs retain distinct cell surface phenotypes following maturation.

In the present study we have identified a distinct CD13+ DC population in bovine spleens that is phenotypically unique compared to other peripheral blood, lymph node, and afferent lymph DCs and confirmed its effectiveness in antigen presentation in both the allogeneic T-cell stimulation reaction and the induction of T-cell recall responses. Unlike the two subsets

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**TABLE 2. Comparison of bovine DC surface phenotypes**

| Surface molecule | Spleen DCs | Peripheral blood DCs | Afferent lymph DC subset 1 | Afferent lymph DC subset 2 |
|------------------|-----------|----------------------|---------------------------|---------------------------|
| MHC class II     | ++        | ++                   | +                         | +                         |
| CD1b             | –         | –                    | –                         | –                         |
| CD2              | –         | –                    | –                         | –                         |
| CD4              | –         | –                    | –                         | –                         |
| CD8              | –         | –                    | –                         | –                         |
| CD11a            | ++        | ++                   | +                         | –                         |
| CD11b            | ±         | +                    | ±                         | ±                         |
| CD11c            | ++        | +                    | ±                         | +                         |
| CD13             | ++        | –                    | +                         | –                         |
| CD21             | –         | –                    | –                         | – or +                    |
| CD45RO           | –         | –                    | +                         | +                         |
| CD80             | ±         | ±                    | +                         | +                         |
| CD86             | –         | –                    | +                         | +                         |
| CD172a           | +         | +                    | –                         | +                         |
| CD205            | ++        | +                    | ++                        | +                         |

* Intensities of monoclonal antibody binding are indicated as – (negative), ± (weak), + (positive), or ++ (strong positive), according to the conventions of Howard and Hope (13). In this study, these symbols are defined as follows: ++, ≥60% of cells were positive; +, 15 to 59% of cells were positive; ±, <15% were positive; and –, cells were negative.

* Splenic and peripheral blood DCs were analyzed as uncultured cells using three-color flow cytometry.

* Data are from references 12 and 13. Uncultured cells were examined.

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**FIG. 6.** Splenic and peripheral blood DCs display distinct cell surface phenotypes. Uncultured DCs were analyzed by flow cytometry. Levels of expression of CD13, CD11b, CD11c, and CD205 are indicated by the solid line. The background of secondary antibody binding is indicated by the light gray dotted lines.
of bovine afferent lymph DCs, which differ in surface pheno-
type, including in the expression of CD11a, CD13, and
CD172a, there was no definitive evidence of multiple splenic
DC subsets when the cells were examined either prior to or
following activation. However, minor subsets not detected in
normal spleens from healthy animals may well be identified if
there is specific expansion or activation by delivery of antigens,
including the blood-borne pathogens *Anaplasma* and *Babesia*,
to the spleen. Determining how splenic DCs are activated and
migrate following the initial encounter with the pathogen and
how priming in the spleen affects T-cell trafficking represent
the next challenges for better understanding immunity against
these important pathogens.

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REFERENCES

1. Armitage, R. J., W. C. Fanslow, L. Stockynch, T. A. Sato, K. N. Clifford, B. M. Macch, D. M. Anderson, S. D. Gimpe, T. Davis-Smith, C. R. Maliszewski, E. A. Clark, C. A. Smith, K. H. Grabstein, D. Cosman, and M. K. Spriggs. 1992. Molecular and biological characterization of a murine ligand for CD40. Nature 357:80–82.

2. Banchereau, J., J. Fay, V. Pascual, and A. K. Palucka. 2003. Dendritic cells: controllers of the immune system and a new promise for immunotherapy. Novartis Found. Symp. 252:226–235.

3. Biragyn, A., M. Surenhu, D. Yang, P. A. Ruffini, B. A. Hamius, E. Klyushnenkova, J. J. Oppenheim, and L. W. Kwak. 2001. Mediators of innate immunity that target immature, but not mature, dendritic cells induce antitumor immunity when genetically fused with nonimmunogenic tumor antigens. J. Immunol. 167:6644–6653.

4. Brown, W. C., and G. H. Palmer. 1999. Designing blood-stage vaccines against *Babesia bovis* and *B. bigemina*. Parasitol. Today 15:275–281.

5. Brown, W. C., T. C. McGuire, D. Zhu, H. A. Levin, J. Sosnow, and G. H. Palmer. 2001. Highly conserved regions of the immunodominant major surface protein 2 of the genogroup II ehrlichial pathogen *Anaplasma margina*are rich in naturally derived CD4+ T lymphocyte epitopes that elicit strong recall responses. J. Immunol. 166:1114–1124.

6. Campbell, D. J., and E. C. Butcher. 2002. Rapid acquisition of tissue-specific homing phenotypes by CD4+ T cells activated in cutaneous or mucosal lymphoid tissues. J. Exp. Med. 195:135–141.

7. Edwards, C. P., and A. Aruffo. 1993. Current applications of COS cell-based transient expression systems. Curr. Opin. Biotechnol. 4:558–563.

8. Engwerda, C. R., I. Beatrice, and F. H. Amante. 2005. The importance of innate immunity that target immature, but not mature, dendritic cells induce antitumor immunity when genetically fused with nonimmunogenic tumor antigens. J. Immunol. 167:6644–6653.

9. Gliddon, D. R., J. C. Hope, G. P. Brooke, and C. J. Howard. 2004. DEC-205 expression on migrating dendritic cells in afferent lymph. Immunology 111:262–272.

10. Heussler, V. T., M. Eichhorn, and D. A. Dobbelraer. 1992. Cloning of a full-length cDNA encoding bovine interleukin 4 by the polymerase chain reaction. Gene 114:273–278.

11. Hirano, A., W. C. Brown, W. Trigona, W. Tso, and D. M. Estes. 1998. Kinetics of expression and subset distribution of the TNF superfamily member CD40 ligand and Fas ligand on T lymphocytes in cattle. Vet. Immunol. Immunopathol. 64:251–263.

12. Howard, C. J., P. Sopp, J. Brownlie, L. S. Kwong, K. R. Parsons, and G. Taylor. 1997. Identification of two distinct populations of dendritic cells in afferent lymph that vary in their ability to stimulate T cells. J. Immunol. 159:5372–5382.

13. Howard, C. J., and J. C. Hope. 2000. Dendritic cells, implications on function from studies of the afferent lymph veiled cell. Vet. Immunol. Immunopathol. 77:1–13.

14. Howard, C. J., B. Charleston, S. A. Stephens, P. Sopp, and J. C. Hope. 2004. The role of dendritic cells in shaping the immune response. Anim. Health Res. Rev. 5:191–195.

15. Inaba, K., W. W. Swiggard, M. Inaba, J. Meltzer, A. Mirza, T. Sasagawa, M. C. Nussenzweig, and R. M. Steinman. 1995. Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145. I. Expression on dendritic cells and other subsets of mouse leukocytes. Cell. Immunol. 165:148–156.

16. Jones, E. W., R. B. Norman, I. O. Kliewer, and W. E. Brock. 1968. *Anaplasma margina* infection in splenectomized calves. Am. J. Vet. Res. 29:523–533.

17. Kantele, A., J. Zivny, M. Hakkinnen, C. O. Elson, and J. Mestecky. 1999. Differential homing commitments of antigen-specific T cells after oral or parenteral immunization in humans. J. Immunol. 162:5173–5177.

18. Losos, G. J. 1986. Babesiosis, p. 41–46. In G. J. Losos (ed.), Infectious diseases of domestic animals. Churchill-Livingston Inc., New York, N.Y.

19. Maliszewski, C. R., K. Grabstein, W. C. Fanslow, R. Armitage, M. K. Spriggs, and T. A. Sato. 1993. Recombinant CD40 ligand stimulation of murine B cell growth and differentiation: cooperative effects of cytokines. Eur. J. Immunol. 23:1044–1049.

20. McInhinney, R. A. 2004. Generation and use of epitope-tagged receptors. Methods Mol. Biol. 259:91–98.

21. Mora, J. M., R. M. Bono, N. Manjunath, W. Wening, L. L. Cavanagh, M. Rosemblatt, and U. H. Von Andrian. 2003. Selective imprinting of gut-homing T cells by Peyers patch dendritic cells. Nature 424:885–93.

22. Mwangi, W., W. C. Brown, and G. H. Palmer. 2000. Identification of fetal liver tyrosine kinase 3 (flt3) ligand domain required for receptor binding and function using naturally occurring ligand isoforms. J. Immunol. 165:6966–6974.

23. Mwangi, W., W. C. Brown, H. A. Levin, C. J. Howard, J. C. Hope, T. V. Baseler, P. Caplai, J. Abbott, and G. H. Palmer. 2002. DNA-encoded fetal liver tyrosine kinase 3 ligand and granulocyte macrophage-colony-stimulat-
ing factor increase dendritic cell recruitment to the inoculation site and enhance antigen-specific CD4+ T cell responses induced by DNA vaccina-
tion of outbred animals. J. Immunol. 169:3837–3846.

24. Mwangi, W., W. C. Brown, G. A. Splitter, Y. Zhuang, K. Kegerreis, and G. H. Palmer. 2005. Enhancement of antigen acquisition by dendritic cells and MHC class II-restricted epitope presentation to CD4+ T cells using VP22 DNA vaccine vectors that promote intercellular spreading following initial transfection. J. Leukoc. Biol. 78:401–411.

25. Norimine, J., and W. C. Brown. 2005. Intrahaplotype and interhaplo-type pairing of bovine leukocyte antigen DQA and DOB molecules generate functional DNA molecules important for priming CD4+ T-lymphocyte re-
sponses. Immunogenetics 57:750–762.

26. Palmer, G. H., F. R. Rurangirwa, K. M. Kocan, and W. C. Brown. 1999. Molecular basis for vaccine development against the ehrlichial pathogen *Anaplasma margina*. Parasitol. Today 15:281–286.

27. Spriggs, M. K., K. J. Mest, D. Stockb, K. N. Clifford, B. M. Macduff, T. A. Sato, C. R. Maliszewski, and W. C. Fanslow. 1992. Recombinant human CD40 ligand stimulates B cell proliferation and immunoglobulin E secretion. J. Exp. Med. 167:1543–1550.

28. Storset, A. K., S. Kalberg, I. Berg, P. Boysen, J. C. Hope, and E. Disen. 2004. NKP46 defines a subset of bovine leukocytes with natural killer cell characteristics. Eur. J. Immunol. 34:669–676.

29. Trombetta, E. S., and J. Mollman. 2005. Cell biology of antigen processing in vitro and in vivo. Annu. Rev. Immunol. 23:975–1028.