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# B-1-like cells exist in sheep. Characterization of their phenotype and behaviour

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## SUMMARY

Two populations of B lymphocytes, B-1 (CD5$^+$ and/or CD11b$^+$) and B-2 (CD5$^-$ and CD11b$^-$) cells have been described. In mice, which is the species of reference for B-1 and B-2 cell studies, these two subsets present different developmental schemes, phenotypes, antibody repertoires, localization and behaviours. Interestingly, in sheep, B cells rearrange their immunoglobulin (Ig) loci around the neonatal period, similarly to murine B-1 cells. However, the phenotype of the sheep B cells has not been characterized with regards to their developmental pathway. In this report, we show that two sheep B-cell subsets can be distinguished on the basis of CD11b expression. Relative to CD11b$^-$ B cells, the CD11b$^+$ B cells frequently co-express CD5, CD11c, higher levels of surface IgM (sIgM), show larger cell size and higher cell-cycling activity, and thus present a B-1-like phenotype. However, unlike murine B-1 cells, sheep B-1 like cells mainly localize in blood, display a higher propensity to spontaneous apoptosis relative to B-2-like cells, and proliferate after sIgM stimulation. Our data show that despite neonatal immunoglobulin loci rearrangements, sheep B cells do not all express a B-1-like phenotype. However, B-1-and B-2-like cells co-exist and present phenotypic and behavioural specificities. Nevertheless, sheep B-1-and B-2-like cells differ from the murine B-1 and B-2 cells in their cell behaviour. These subsets can thus not be considered as true homologues among species.

## INTRODUCTION

Two schemes of B-cell development have been described in different species. In the type 1 pathway, the immunoglobulin (Ig) locus rearranges early in ontogeny during a narrow period of development. Afterwards, the definitive pool of B cells expands by a self-renewing mechanism. This scheme applies to mouse B-1,[1] rabbit B,[2] bird B[3] and sheep B lymphocytes.[4] In the type 2 pathway, the immunoglobulin repertoires are continuously generated from precursors in the bone marrow during the whole life. This type-2 scheme applies to mouse conventional B-2 cells and to most human adult B lymphocytes.[5] Thus at least two B-cell lineages, B-1 and B-2 lymphocytes, co-exist in the mouse. This assessment is supported by experiments in which adoptive transfers of donor B lymphocytes into irradiated recipients differentially reconstitute each subset.[6]

In addition to different developmental characteristics, murine B-1 cells demonstrate phenotypic and functional properties that distinguish them from murine B-2 cells (reviewed by Hayakawa *et al.*[7]) (i) B-1 cells display high levels of surface IgM, low levels of IgD, of B220 (CD45RA), and of Mac-1 (CD11b/CD18), a β2 integrin normally associated with the myelomonocytic lineage; the CD5 marker allows the further distinction of B-1 cells into two subpopulations: a CD5$^+$ CD11b$^+$ IgM$^{high}$ IgD$^{low}$-predominant subset named B-1a, and a CD5$^-$ CD11b$^+$ IgM$^{high}$ IgD$^{low}$ minor 'sister' population named B-1b that otherwise appears to be identical to the B-1a subset; (ii) B-1 cells are preferentially represented in the peritoneal cavity and are largely absent from blood and lymph nodes; (iii) B-1 cells are larger than conventional B-2 lymphocytes; (iv) B-1 cells produce polyreactive antibodies mainly directed against autoantigens and bacterial antigens; (v) ligation of surface IgM (sIgM) induces apoptosis of B-1 cells and proliferation of B-2 cells;[8] (vi) B-1 cells show a higher capacity for cell survival *ex vivo*;[9] (vii) finally, B-1 cells are often involved in B-cell leukaemias and their number increases in autoimmune diseases.

It is commonly accepted that B cells expressing CD5 and/or CD11b should be named B-1 cells both in humans and mice.[10] CD5$^+$ B cells have also been described in cows,[11] rats[12] and rabbits.[2] Interestingly in rabbits, most (95%) of the blood B

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Abbreviations: f, follicular zone; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FSC, forward scatter; Ig, immunoglobulin; sIg, surface immunoglobulin; LPS, lipopolysaccharide; mAb, monoclonal antibody; MFI, mean fluorescence intensity; mrz, marginal zone; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; PMA, phorbol myristate acetate; SSC, side scatter; TRITC, rhodamin.

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cells express CD5[2] and their primary repertoires are made in the appendix around the neonatal period, an observation consistent with a type 1 developmental pathway.[13] It was then suggested that the type 1 B-cell development may be associated with CD5 expression across species.[13]

In sheep, the type 1 pathway dominates B-cell development: the Ig loci rearrange at mid gestation in the spleen and no new rearrangement occurs afterwards.[14] Subsequently, expansion of the B-cell population and positive selection leads to constitution of the pre-immune antibody repertoire in the ileal Peyer's patches;[4] removal of the ileal Peyer's patches soon after birth dramatically reduces the immunoglobulin level in the blood.[15] It appears from several reports that the major B-cell phenotype in sheep is not in total accordance with the type 1 pathway. For example, similarly to murine B-1 cells, sheep B lymphocytes do not express IgD,[16] but unlike murine B-1 cells, sheep blood B cells rarely express the CD5 molecule.[14,17,18] This suggests that unlike in rabbits and mice, the type 1 development in sheep is not associated with expression of CD5. Besides, we recently reported that bovine leukaemia virus (BLV) infection in sheep induces the exclusive expansion of a CD11b$^+$ B-cell population. The virus protects this population from undergoing apoptosis.[19] We thus aimed at further characterizing the CD11b$^+$ B cells in normal sheep in terms of their phenotype and behaviour. In this report we show that the CD11b$^+$ B cells correspond to a B-1-like phenotype in sheep blood with expression of CD5, CD11c, high IgM, large cell size and a relatively higher cell-cycling activity. However, differences in cellular responses between murine B-1/B-2 cells and sheep B-1/B-2-like cells suggest that although these cells share common phenotypic traits, they can not be considered as true homologues.

## MATERIALS AND METHODS

*Animals*

Four pre-alpine castrated sheep used in follow-up studies have been housed for 6–8 years at the Ecole Nationale Vétérinaire d'Alfort (France). The other pre-alpine sheep of different ages were healthy females slaughtered at the Institut National pour la Recherche Agronomique, Jouy-en-Josas, France.

*Monoclonal antibodies (mAbs) and immunophenotyping*

The mAbs used in this study were an anti-CD21 that labels most B lymphocytes in sheep (the IgM, DU2-104),[14] an anti-CD5 (the IgG1, CC17),[20] two anti-CD11b (the IgG1, CC125 and the IgG2a, ILA-130),[20] an anti-CD11c (the IgG1, OM1),[21] an anti-μ chain (the IgG1, 1H4)[17] and an anti-CD1 (the IgG1, VC13).[20] Cells were incubated for 30 min in mixes of 50 μl hybridoma supernatants. After wash, they were further incubated as described by Schwartz-Cornil *et al.*[22] with a 1:50 dilution of fluorochrom-conjugated $F(ab')_2$ goat antibody directed against specific murine isotypes (Caltag Laboratories, San Francisco, CA).

*Leucocyte preparations*

Peripheral blood mononuclear cells (PBMCs) were purified by percoll gradient centrifugation as described by Cornil *et al.*[23] Leucocytes from lymphoid tissues (spleen, lymph nodes, Peyer's patches, tonsils) were simply obtained by fine mincing followed by an enzymatic treatment.[24]

*Immunohistochemistry*

Sheep lymphoid organs were cryostat sectioned and mounted onto microscope slides. The rehydrated sections were pre-incubated in 10% goat serum, and reacted for 30 min with 100 μl of the DU2-104 supernatant and a 1:100 dilution of the ILA-130 antibody ascites. After three washes, the sections were incubated for 30 min with a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-mouse IgM (Caltag Laboratory) and with a 1:50 dilution of a rhodamin (TRITC)-conjugated $F(ab')_2$ goat anti-mouse IgG2a (Caltag Laboratory).

Ex vivo *cultures of PBMCs and apoptosis detection*

PBMCs ($5 \times 10^6$ cells per ml) were seeded in RPMI-1640 plus 10% fetal calf serum (FCS) and incubated for 48 hr in 5% $CO_2$ at 37°. PBMCs were then labelled for detection of CD21 and CD11b expression using the DU2-104 and the CC125 mAbs followed by phycoerythrin (PE)-conjugated $F(ab')_2$ goat anti-mouse IgM antibody and tricolour-conjugated $F(ab')_2$ goat anti-mouse IgG1 antibody. They were reacted with FITC-conjugated annexin V (Boehringer Mannheim, Mannheim, Germany) to detect apoptotic cells according to the manufacturer's recommendations.

*Cell-cycle analyses*

Fresh or cultured PBMCs were stained for detection of CD21 and CD11b using the DU2-104 and CC125 mAbs as for apoptosis detection and then permeabilized using 70% cold ethanol for 10 min at −20°. After a 30-min treatment with 20 μg/ml RNAse A at 37°, the cells were incubated with a $1:10^6$ dilution of Sytox Green Nucleic Acid Stain (Molecular Probes, Eugene, OR) and analysed using flow cytometry.

*B-cell activation studies*

For cell-cycling analyses and apoptosis detection, PBMCs were incubated for 48 hr in RPMI plus 10% FCS plus $7 \cdot 5 \times 10^{-5}$ M β-mercapto-ethanol, with 10 μg/ml purified rabbit immunoglobulins to sheep μ-chain (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD). For modulation of CD11b expression, PBMCs were incubated for 24–72 hr with 10–100 ng/ml phorbol myristate acetate (PMA) (Sigma Aldrich, St Louis, MO), with 10 to 100 μg/ml rabbit anti-sheep μ-chain, with 1 to 100 μg/ml ionomycin (Sigma), with 1–50 μg/ml lipopolysaccharide (LPS) (Sigma) alone or in combination, with 1000 UI/ml recombinant human interleukin-4 (IL-4; Genzyme, Cambridge, MA) and 100 ng/ml recombinant human tumour necrosis factor-α (TNF-α, Genzyme).

## RESULTS

### Expression of CD11b on sheep blood B cells

We and others have reported that some sheep blood B cells express CD11b. [19,25] However, the extent of CD11b expression on sheep blood B cells and how it may vary with age, time, and between individuals needs to be determined. We found that CD11b is detected on a large proportion of the blood B cells and that it is expressed at a lower level on B cells than on monocytes (compare top to bottom right panel, Fig. 1a). Analyses on 5-month to 8-year-old sheep showed that between 35 and 87% of the blood B cells express CD11b (mean ± SD: 62% ± 16% from 11 sheep, data not shown). An age of between

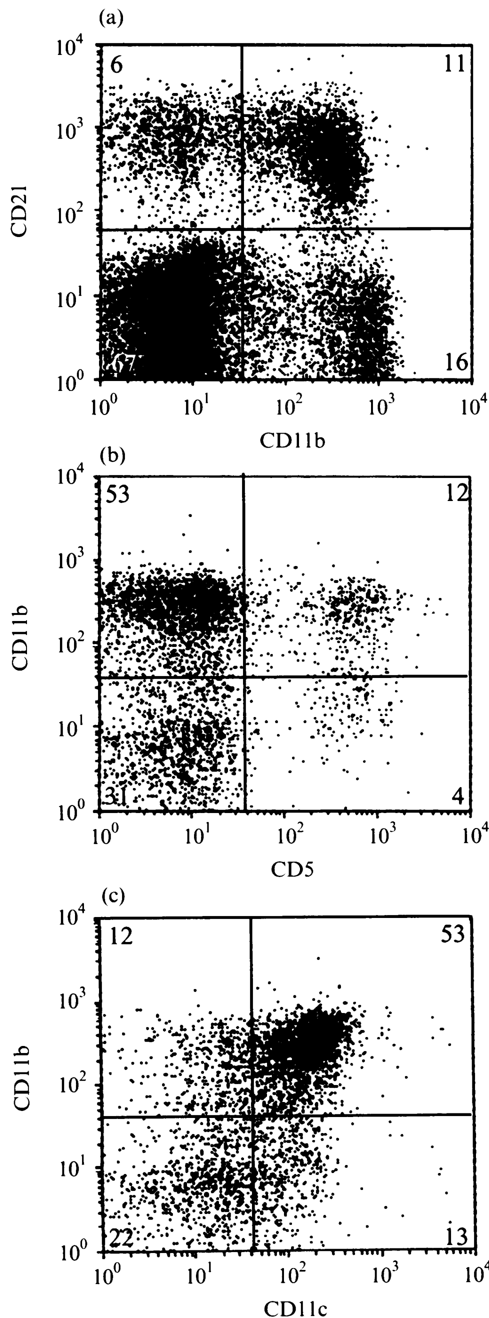


**Figure 1.** Expression of CD11b, CD5 and CD11c on sheep PBMCs. (a) PBMCs were labelled for detection of CD21 and CD11b using the DU2-104 mAb followed by a PE-conjugated $F(ab')_2$ goat anti-mouse IgM and the ILA-130 mAb followed by a FITC-conjugated $F(ab')_2$ goat anti-mouse IgG2a. Cells were then processed for fluorescence activated cell sorter (FACS) analysis and the percentage of cells in each subset is shown. (b) Detection of CD5 was performed with the CC17 mAb followed by a tricolour-conjugated $F(ab')_2$ goat anti-mouse IgG1 antibody on PBMCs labelled for CD21 and CD11b detection as described above. The gated CD21-positive cells (B cells) were analysed for CD5 and CD11b expression. (c) Detection of CD11c was carried out with the OM1 mAb as in B.

5 months and 8 years does not seem to influence the proportion of $CD11b^+$ B cells in the blood. We also found that the percentage of $CD11b^+$ B cells varies within a $\pm 10$ range between blood samples from the same animal (see sheep nos. 126 and 128, Table 1).

### Phenotypic analysis of sheep blood $CD11b^+$ B cells

CD11b is expressed on B-1 cells in mice. We thus tested whether other surface molecules and morphological parameters associated with the B-1 lineage are found on sheep blood $CD11b^+$ B cells.

The most classic molecule found on B-1 cells is the CD5 molecule. Triple stain for the CD21, CD5 and CD11b molecules were performed three times on blood PBMCs from four sheep (Fig. 1b, Table 1). Overall, the percentage of $CD5^+$ B cells was extremely variable and laid between 16% and 82% of the B cells, depending on the sheep and on the time of the analysis. The proportion of B cells doubly positive for CD5 and CD11b expression varied between 12 and 65% of the B cells. A significant percentage of the $CD5^+$ $CD11b^-$ B cells were detected and oscillated between 2 and 27% of the B cells; however, the CD5 expression was more frequently found on $CD11b^+$ cells than on $CD11b^-$ B cells as 64% to 92% of the $CD5^+$ B cells were CD11b positive (Table 1). The level of expression of the CD5 molecule that we detected on B cells greatly varied between analyses for the same sheep and could sometimes appear very weak. Overall, CD5 expression is not consistently found on sheep B cells but it is usually found associated with CD11b.

A β2 integrin α-chain, CD11c, has been described as being associated with the CD5 phenotype on human B-1 cells from chronic lymphocytic leukaemias.[26] We also encountered the expression of CD11c on most sheep blood B cells (Fig. 1c): 38 to 78% of the B cells were doubly positive for CD11b and CD11c, and some B cells (6 to 28%) were $CD11c^+CD11b^-$.

A cardinal feature of the murine B-1 cells is the high expression of IgM. Sheep $CD11b^+$ B cells consistently showed much higher levels of IgM expression than the $CD11b^-$ B lymphocytes. Based on analyses of four sheep, $76\% \pm 9\%$ of the $CD11b^+$ B cells and $25\% \pm 10\%$ of the $CD11b^-$ B cells expressed high levels of IgM, respectively (Fig. 2a). In addition, there was a strong correlation between the intensities of CD11b and sIgM labelling in the B-cell population ($r^2 = 0{\cdot}94$, Fig. 2b), indicating that these molecules may be co-regulated. We also found that a high level of sIgM was associated with CD11b, but not with CD5 expression (Fig. 2c).

Murine B-1 cells are large and granular as compared with B-2 lymphocytes. As depicted in Fig. 3, the forward scatter (FSC) to the side-angle light scatter (SSC) of the $CD11b^+$ and $CD11b^-$ B cells are clearly different and show that the $CD11b^+$ B cells are at least twice as large and granular as the $CD11b^-$ B cells.

Altogether these phenotypic analyses show the CD11b molecule characterizes blood B lymphocytes with frequent co-expression of CD5, high IgM expression and large cell size. The blood $CD11b^+$ B cells thus share several phenotypic traits with murine B-1 cells. Consequently, on the basis of phenotype, $CD11b^+$ B cells in sheep will be referred to as B-1-like cells.

**Table 1.** Representation of the CD11b$^+$ and CD5$^+$ B-cell subsets in sheep blood

| | Sheep* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 126 | | 128 | | 185 | | 190 | |
| Date | CD11b$^+$ | CD5$^+$ | CD11b$^+$ | CD5$^+$ | CD11b$^+$ | CD5$^+$ | CD11b$^+$ | CD5$^+$ |
| July 1996 | 58† | 50‡ | 60 | 30 | 62 | 45 | 84 | 57 |
| November 1996 | 44 | 33 (70)§ | 79 | 82 (79) | 67 | 76 (64) | 84 | 67 (89) |
| November 1997 | 64 | 16 (75) | 80 | 27 (92) | 70 | 18 (83) | 87 | 25 (92) |

*Sheep identification.
†Percentage of CD11b$^+$ B cells among B cells.
‡Percentage of CD5$^+$ B cells among B cells. §()% of CD11b$^+$ B cells among CD5$^+$ B cells.

**Organ distribution of the CD11b$^+$ B cells**

So far, we found that CD11b$^+$ B cells are largely represented in blood. As B-1 cells in mice have a distinct anatomic distribution, CD11b$^+$ B cells may localize to specific lymphoid compartments. As presented in Table 2, rare CD11b$^+$ B lymphocytes – from 14 to 2% of the B cells – were encountered in lymph nodes, Peyer's patches and tonsils. A significant number of CD11b$^+$ B cells was seen in spleen (24%$\pm$10% of the B cells). This distribution indicates that CD11b expression in B cells is associated with blood localization, as the spleen is a blood filter. Similarly, very few CD5$^+$ B cells were detected in the spleen and lymph nodes, and represented less then 10% of the B cells (data not shown). Immunohistochemical analyses of frozen spleen sections showed that the doubly positive CD21$^+$CD11b$^+$ cells were localized to the marginal zone (Fig. 4). Such a distribution in the spleen has been described for rat and mouse B-1 cells.[27]

These results show that CD11bь+ B cells are rarely found in lymph nodes but are mainly localized in the blood and spleen. Overall, despite a type 1 developmental pathway, a large proportion of the sheep B cells, i.e. the lymph B cells, do not express the B-1-like phenotype.

**CD11b$^+$ B cells show a relatively higher cell-cycling activity and they proliferate following triggering by sIgM**

As sheep CD11b$^+$ B cells show distinct phenotypic characteristics in common with murine B-1 lymphocytes, we asked whether they would also show peculiar biological behaviours. Ligation of sIgM on murine B-1 cells leads to apoptosis, whereas it induces B-2 cells to enter into the S phase of the cell cycle.[8] Sheep PBMCs were stimulated for 48 hr with rat anti-sheep IgM and analysed for cell-cycling activity. Very few cells from freshly harvested PBMCs were cycling, but the CD11b$^+$ B-cell population showed a tendency to contain more cycling cells (Table 3). By contrast, the *ex vivo* PBMC culture led to activation of cellular proliferation and cross-linking of the sIgM-induced cell entry into the cell cycle in both B-cell subpopulations (Table 3). Analysis of the subdiploid population and labelling with annexin V showed that ligation of the sIgM did not induce apoptosis in both the CD11b$^+$ and CD11b$^-$ subsets (data not shown).

These experiments show that fresh or cultured CD11b$^+$ B cells are more engaged into the cell cycle than are CD11b$^-$ B cells, a property that is also described for murine B-1 cells. In spite of their phenotypic similarities with mouse B-1 cells, sheep-blood CD11b$^+$ B cells respond differently to mouse B-1 cells to the sIgM ligation, i.e. they do not enter apoptosis but proliferate.

**Table 2.** CD11b$^+$ B cells in lymphoid organs

| Tissues | % CD11b$^+$ B/B cells* |
|---|---|
| Blood | 66$\pm$10 |
| Spleen | 24$\pm$10 |
| Lymph nodes | |
| Inguinal | 14$\pm$2 |
| Mesenteric | 6$\pm$1 |
| Tonsils | 7$\pm$2 |
| Peyer's patches | |
| Ileal | 2$\pm$0·5 |
| Jejunal | 3$\pm$0·1 |

*Percentage of CD11b$^+$ B cells among B cells. Data are mean$\pm$SD from three 1-year-old female sheep.

**CD11b$^+$ B cells show a higher propensity to spontaneous apoptosis *in vitro***

Murine B-1 cells are more resilient to *in vitro* cell culture than are B-2 cells.[9] We thus compared the survival of CD11b$^+$ and CD11b$^-$ B cells *ex vivo*. Table 4 shows that CD11b$^+$ B cells present a lower level of *ex vivo* survival than CD11b$^-$ B cells after a 48 hr culture. This finding indicates that sheep B-1- and B-2-like cells present survival properties that are opposite to the ones obtained with murine B-1 and B-2 cells.

**Classical B-cell activation does not trigger CD11b expression**

As sheep B-1-like cells present some features of activated cells (large cell size, higher cell-cycling activity), we hypothesised that activation of sheep B cells may induce or enhance CD11b expression. Unfortunately, neither PMA, rat anti-sheep sIgM, ionomycin, LPS, (alone or in combination) recombinant human IL-4, recombinant human TNF-α, induced neither CD11b nor CD5 on B cells from PBMCs cultures (data not shown). This negative result suggests that CD11b expression on B cells is not associated with 'classic' B-cell activation.

## DISCUSSION

In this study, we have shown that although the majority of sheep B cells follow a type 1 development, they do not all

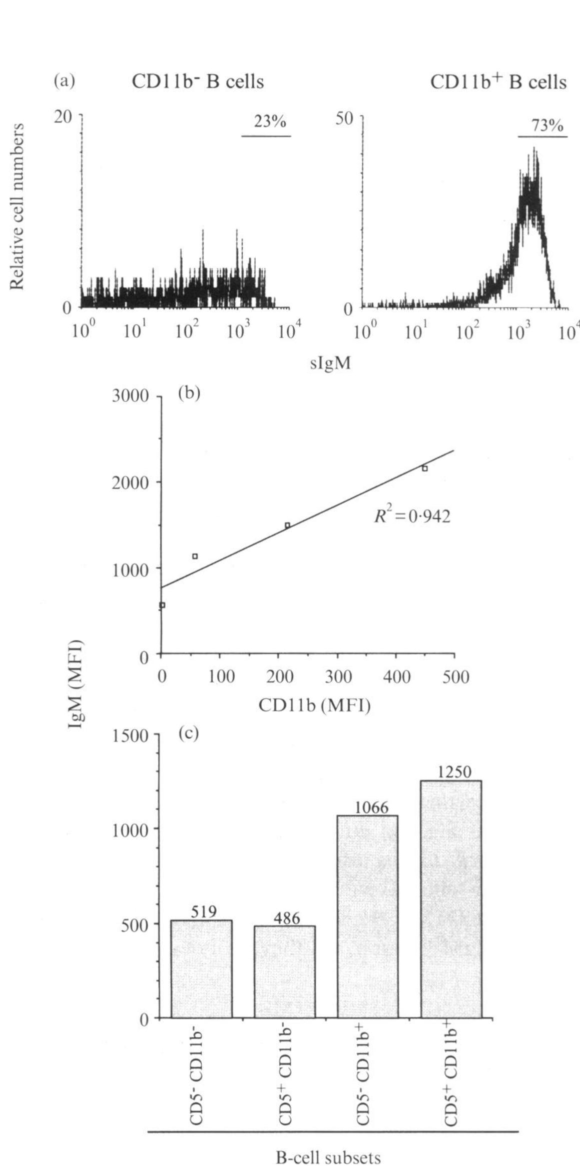


**Figure 2.** Expression of sIgM on $CD11b^+$ and $CD11b^-$ B cells. PBMCs were labelled for CD21 and CD11b expression as in Fig. 1, and further labelled for sIgM detection using the 1H4 mAb followed by a tricolour $F(ab')_2$ goat anti-mouse IgG1 antibody. (a) The gated $CD11b^+$ and $CD11b^-$ B cells were analysed for IgM expression and the percentage of cells showing high expression is indicated. (b) The B population of one sheep was divided into four subsets showing increasing mean fluorescence intensities for CD11b labelling (MFI, logarithmic arbitrary units, $x$-axis), i.e. CD11b negative B cells (MFI = 1) and CD11b-positive B cells (MFI = 57, 215, 449). On the $y$-axis, the MFI corresponding to the sIgM labelling of the four B-cell subsets are reported. A correlation slope was derived ($R^2 = 0{\cdot}942$). (c) PBMCs were labelled for CD11b, CD5 and sIgM detection. The MFI of the different B-cell subsets are indicated.

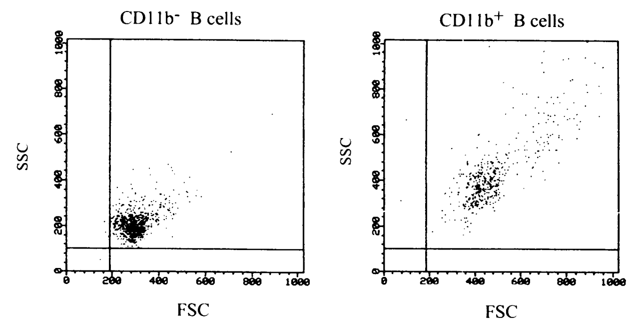


**Figure 3.** Cell size and granularity of $CD11b^+$ and $CD11b^-$ B cells. PBMCs were labelled for CD21 and CD11b detection as in Fig. 1. The gated $CD11b^+$ and $CD11b^-$ B cells were analysed for their FSC and SSC light-angle emission. The FCS and SSC values are size and granularity parameters, respectively.

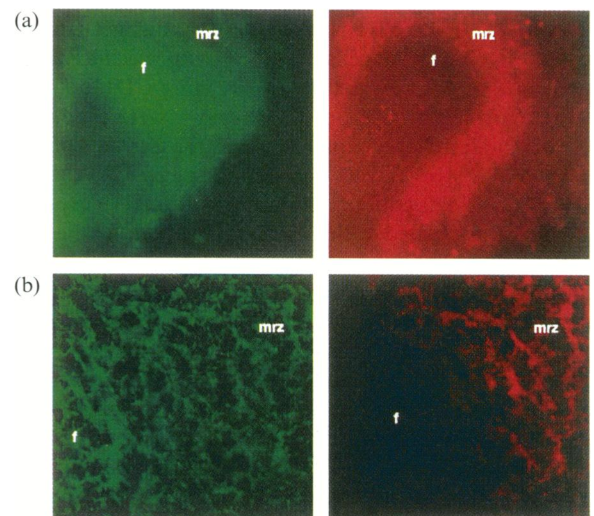


**Figure 4.** Localization of $CD11b^+$ B cells in the spleen. Frozen slices of spleen were reacted with the DU2-104 (CD21) and the ILA-130 mAb (CD11b) followed by the FITC-conjugated $F(ab')_2$ goat anti-mouse IgM and the TRITC-conjugated $F(ab')_2$ goat anti-mouse IgG2a. (a) ×8 magnification; (f) and (mrz) stand for follicular and mantle zone area; (b) ×32 magnification.

express a B-1-like phenotype, by contrast with rabbit B and murine B-1 cells. However, sheep B cells can still be phenotypically separated into two populations (on the basis of CD11b expression), referred to as sheep B-1- and B-2-like cells that show distinct cell behaviours. Nevertheless, these cells do not behave like murine B-1 and B-2 cells, indicating that they can not be considered to be homologues.

On the basis of developmental characteristics, $CD5^+$ and/or $CD11b^+$ B cells do not refer to similar cells among species and consequently, inter-species extrapolations are abusive. In the mouse, which is the species of reference for B-1 and B-2 cell studies, the scheme of B-cell development is peculiar and does not apply to other species. Most reports support that the concept that murine B-1 and B-2 cells belong to two independent lineages that follow a type 1 and type 2 developmental pathway, respectively.[6,10,28,29] In sheep, the B-cell development is essentially similar to that in murine B-1.[30] The B-cell lymphopoietic activity in bone marrow, if it exists, is very low. As removal of most of the ileal Peyer's patches around the neonatal period leads to a dramatic reduction of the circulating B cells and the immunoglobulin level,[31] it is likely that both $CD11b^+$ and $CD11b^-$ B cells follow a type 1 developmental pathway relying on Peyer's patches. Yet, there is the remote possibility that sheep B-1-

**Table 3.** Cell-cycle activity in $CD11b^+$ and $CD11b^-$ B cells

| B-cell subsets | Experiment 1 | | | Experiment 2 | | |
|---|---|---|---|---|---|---|
| | $t=0$ | $t=48$ hr | +anti IgM, $t=48$ hr | $t=0$ | $t=48$ hr | +anti-IgM, $t=48$ hr |
| $CD11b^+$ | 1·3* | 5·8 | 21 | 2·7 | 8 | 12·5 |
| $CD11b^-$ | 0·9 | 3·9 | 11 | 1·7 | 2·5 | 12 |

*Percentage of cells in S phase among freshly harvested B cells from sheep 190 ($t=0$), 48 hr cultured B cells ($t=48$ hr), B cells treated for 48 hr with 10 μg/ml anti-IgM (+anti-IgM, $t=48$ hr).

**Table 4.** Survival of $CD11b^+$ and $CD11b^-$ B cells in short-term cultures

| B-cell subsets | Sheep* | | |
|---|---|---|---|
| | 126 | 185 | 190 |
| $CD11b^+$ | $34\pm6$† | $35\pm9$ | $39\pm5$ |
| $CD11b^-$ | $56\pm10$ | $60\pm10$ | $62\pm9$ |

*Sheep identification.

†Percent of annexin V negative cells after 48 hr cultures. Results are means and SD from seven independent experiments. A comparison between the cell survival in the $CD11b^+$ and $CD11b^-$ subsets reveals a $P<0{\cdot}005$ using a Student's $t$-test.

and B-2-like cells arise at different times and places during ontogeny, before the Peyer's patch colonization. In any event, B-1- and B-2-like cells in sheep do not follow the same differential developments as murine B-1 and B-2 cells.

If B-1- and B-2-like cells in sheep belong to a single B lineage, it would mean that these phenotypes correspond to two differentiation/activation stages, presenting specific marker expressions and cellular responses. Actually, several findings in this study support the theory that sheep B-1 like cells show a B-cell phenotype associated with cell activation: **1**. $CD11b^+$ B cells are large and granular cells, which is a sign of increased cell metabolism; **2**. the population of $CD11b^+$ B cells is enriched in cells in an S phase, indicating that this population presents a relatively higher cellular activity; **3**. a higher propensity to apoptosis found among $CD11b^+$ B cells is coherent with an activated state;[32–34] **4**. the representation of the $CD11b^+$ B population may vary between analyses for an individual, independently of its age (see Table 1). This means that environmental factors, such as microbial or parasitic infections, may affect the expansion of this population. For instance, infection by *Trypanosoma congolense* leads to the expansion of a $CD5^+CD11b^+$ B-cell population in cattle,[11] a species closely related to sheep. However, the pathway of activation towards expression of the B-1-like phenotype remains to be discovered as common B-cell activators such as PMA, anti-IgM and ionomycin were inefficient to trigger CD11b or CD5 expression. The pathway of activation/differentiation to the B-1-like phenotype may be associated with blood localization. The B-1-like phenotype could be acquired whilst the cells cross the vasculature to enter the blood flow and they may then circulate in blood until death and/or lodge into yet to be defined tissues. Conversely, it is possible that the B-1-like phenotype may reverse to a B-2-like phenotype when the cells enter the lymph node from the blood.

Despite heterogeneous developmental origins, B-1 cells from mice, and B-1 like cells from rats, sheep and humans, present phenotypic features in common, such as expression of CD5, CD11b, CD11c, high levels of sIgM, large cell size, and higher engagement in the cell cycle.[7,35] It is also striking that sheep, rat and mouse B-1 cells localize to the spleen marginal zone.[27] This observation may result from common regulation pathways of genes that may be co-activated in many species. The strong correlation between the level of CD11b and sIgM expression is in favour of this co-regulation hypothesis.

In conclusion, although most sheep B cells follow a type 1 development, they do not all display a B-1-like phenotype. Rather, two B-1-and B-2-like populations exist, presenting phenotypic and behavioural specificities. These findings lead the way to further studies regarding the respective role of the B-1- and B-2-like cells in the ovine immune response.

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