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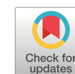
Age-related distribution and dynamics of T-cells in blood and lymphoid tissues of goats



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ABSTRACT

Neonatal mammals have increased disease susceptibility and sub-optimal vaccine responses. This raises problems in both humans and farm animals. The high prevalence of paratuberculosis in goats and the lack of an effective vaccine against it have a strong impact on the dairy sector, and calls for vaccines optimized for the neonatal immune system. We characterized the composition of the T-cell pool in neonatal kids and adult goats and quantified their turnover rates using *in vivo* deuterium labelling. From birth to adulthood, $CD4^+$ T-cells were the predominant subset in the thymus and lymph nodes, while spleen and bone marrow contained mainly $CD8^+$ lymphocytes. In blood, $CD4^+$ T-cells were the predominant subset during the neonatal period, while $CD8^+$ T-cells predominated in adults. We observed that thymic mass and cellularity increased during the first 5 months after birth, but decreased later in life. Deuterium labelling revealed that T-cell turnover rates in neonatal kids are considerably higher than in adult animals.

1. Introduction

The immune system of mammals develops in the protected uterine environment and is still immature in new-born infants (Butler et al., 2009; Chattha et al., 2010a, 2010b; Mohr and Siegrist, 2016; Morein et al., 2007). Immune competence in neonates progresses rapidly after birth. Nevertheless, early after birth the immune system is not yet fully functional, which leads to higher susceptibility to infections and lower vaccine responses in neonates compared to adults (Kollmann et al., 2017; Mohr and Siegrist, 2016; Zens et al., 2017). Humans and farm animals are routinely vaccinated in the first months of life (Harris et al., 2016; Nissen et al., 2017; Pérez de Val et al., 2012; Santema et al., 2011; Windsor, 2015). Neonatal responses to vaccination frequently show limited effectiveness, are short-lived and often require a booster later in life (Siegrist, 2001). To enhance protection against early life-threatening pathogens, such as mycobacteria (Delgado et al., 2013), respiratory syncytial virus (Sacco et al., 2012), among others (Harp et al., 1990; Hunter et al., 2012), novel vaccine formulations and immunization strategies that are optimized for the immune system in early life need to be developed (Mohr and Siegrist, 2016).

The design of effective vaccines and immunization strategies for infants requires a better understanding of the immune system early in life. Most knowledge on the development and kinetics of the immune system comes from studies in mice. Although mouse studies have been extremely instrumental for our understanding of the immune system, the immune system of laboratory mice does not resemble all aspects of that of humans (Beura et al., 2016; Mestas and Hughes, 2004), and is also different from that of ruminants, which are much bigger in size, outbred, have longer life-spans, and are housed under immunologically more challenging circumstances (Bailey et al., 2013; Jolles et al., 2015). Already in 2003, Hein and Gribel reviewed the increasing number of research areas in immunology in which large animals as experimental models offer important advantages (Hein and Griebel, 2003). Large animals are especially relevant in developmental immunology, since in many aspects of fetal development and ontogeny of the immune system there are larger differences between mice and ruminants, than between humans and ruminants. These differences include the duration of gestation, the type of placentation, the stage of fetal development and

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immunecompetence at birth (Cunningham et al., 2001), and the maintenance of the peripheral naive T-cell pool (den Braber et al., 2012). In order to tackle the challenges faced in young children and farm animals, there is thus a great need for studies into the early-life immune system of species other than mice (Davis, 2008; Furman and Davis, 2015; Germain, 2010). In humans, the recent progress in the understanding of the immune system early in life has already led to the identification of vaccine formulations specifically designed for the prenatal and early postnatal periods (Mohr and Siegrist, 2016). A better understanding of the developing immune system of neonates is still lacking for many farm animals.

Paratuberculosis (*Mycobacterium avium* ssp *paratuberculosis*, MAP) and Q fever (*Coxiella burnetii*) have a strong impact on the livestock industry and the dairy goat sector. Following the largest human Q fever epidemic in the Netherlands, which had its source in dairy goat herds, it has been shown that only vaccination can prevent and control Q fever outbreaks in dairy goat farms (Bontje et al., 2016). For paratuberculosis, no effective vaccines are currently commercially available (Park and Yoo, 2016); vaccination performed in young goats limits the clinical signs of infections, but generally fails to prevent infection. Vaccines against paratuberculosis and Q fever are live attenuated or whole-cell vaccines, which are thought to work through activation of αβ T-cells (Faisal et al., 2013). Although ruminants are considered γδ T-cell high species, in which γδ T-cells make up 20–50% of the circulating T-cell pool (Baldwin and Telfer, 2015; Holderness et al., 2013), goats seem to be an exception, since the fraction of γδ T-cells in circulation rapidly decreases the first month after birth, and it is ~5% of the circulating lymphocyte pool in adult goats (Caro et al., 1998). Optimisation of vaccines against paratuberculosis and Q fever in young goats requires a better understanding of the goat adaptive immune system, particularly of the αβ T-cell compartment, during ontogeny.

In this study we provide a comprehensive analysis of the goat αβ T-lymphocyte compartment in neonatal kids, during the first 5 months of life, and compared it to adult goats, 2–6 years of age. We describe the changes in leukocyte numbers and in the distribution of $CD4^+$ and $CD8^+$ T-cells in the thymus, lymph nodes (LN), spleen, blood and bone marrow (BM) with age. Furthermore, we quantified the turnover rate of blood and LN-derived $CD4^+$ and $CD8^+$ T-cells in neonatal kids and adult goats using *in vivo* deuterium labelling.

2. Material and methods

2.1. Goats

Neonatal female White Saanen crossbred dairy goats (N = 24) were purchased from a high health commercial farm, which had a proven history of being free of a number of common caprine infections, including paratuberculosis, caprine arthritic encephalitis and caseous lymphadenitis. From the age of 2 days onwards, the animals were housed at the Department of Farm Animal Health of the Faculty of Veterinary Medicine at Utrecht University, Utrecht, The Netherlands for the duration of one to four-and-a-half months. Adult female goats (N = 34, 2–3 years of age) also purchased from commercial farms were housed at Wageningen Bioveterinary Research, Wageningen, Lelystad, The Netherlands. Additional autopsy material from 5 adult goats (3–6 years of age), and 1 pregnant goat and its 2 fetuses were obtained at the necropsy facility of Wageningen Bioveterinary Research Wageningen, Lelystad, The Netherlands. At necropsy, general macroscopic pathology was performed and samples of the intestinal tissues were tested for the presence of MAP DNA. All samples were confirmed negative.

2.2. Ethics

Animal experiments were approved by the animal experiment commissions of Utrecht University (DEC2014.II.11.085) and Wageningen Bioveterinary Research (permit number AVD401002016580), and were conducted in accordance with the national regulations on animal experimentation.

2.3. In vivo stable isotope labelling

Neonatal goats were sourced in 2 equal groups of 12 animals from two birth cohorts two weeks apart from the same farm. The first group received 3% and the second 2% deuterated water (^2H_2O) (99.8%; Cambridge Isotope Laboratories) for 28 days. The ^2H_2O was mixed with milk replacer in an automated formula milk mixer and dispenser, and animals drank *ad libitum*. Adult goats received 4% ^2H_2O (99.8%) in the drinking water *ad libitum* for 28 days. Urine and plasma sampled from neonatal kids and plasma from adult animals were used to determine deuterium enrichment in the body water during the up- and down-labelling phases. All samples were first frozen and stored at −20 °C until analysis.

2.4. Cell preparation

Animals were sacrificed by intravenous injection of a lethal dose of pentobarbital (Euthasol, AST Farma, Oudewater, The Netherlands) at different time points after start of label administration. Thymus (thoracic and cervical), spleen, left and right pre-scapular lymph nodes (LNs), the humerus and the middle part of the sternum were isolated and weighed. Venous blood was collected from the jugular vein in heparinized Vacutainer (BD Biosciences) tubes prior to injection with pentobarbital. Single cell suspensions from LN were obtained by mechanical disruption of the entire LN, and from thymus, spleen and ileum by mechanically disrupting 2–4 g of tissue. Single cell suspensions from bone marrow (BM) were obtained by flushing the humerus and the sternum. BM cell suspensions were lysed with ACK lysis buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM Na2-EDTA, in H_2O, pH = 7.0) to remove residual erythrocytes. Peripheral blood mononuclear cells (PBMCs) were isolated from blood using Ficoll Plaque (GE Healthcare) density gradient centrifugation. Cell suspensions were further prepared for cell staining and sorting.

2.5. Flow cytometry

Thymocytes, splenocytes, LN, BM, ileum cell suspensions and PBMCs were stained for extracellular markers using CD4-AF647 (clone 44.38, AbD Serotec), CD8-PE (clone 38.65, AbD Serotec), CD14-Viogreen (clone TÜK4, Miltenyi Biotec), and γδ WC1-FITC (clone CC15, Abd Serotec) monoclonal antibodies and 7AAD (BD Biosciences). Cells were analysed on an LSR-Fortessa flow cytometer using FACS Diva software (BD Biosciences).

Viable single lymphocytes were identified based on side scatter and forward scatter characteristics (SSC-A^{low}/FSC-A^{low}) and 7AAD staining (Sup. Fig. 1A). Due to the lack of anti-caprine T-cell specific lineage markers (Davis et al., 2007; Davis and Ellis, 1991), anti-CD14 antibody was used to exclude the potential presence of CD4 or CD8 positive monocytes (Sup. Fig. 1B); $CD4^+$ and $CD8^+$ cells were distinguished within the $CD14^-$ population (Sup. Fig. 1C). Cytospin and Leishman staining performed on sorted lymphocytes confirmed the phenotype of the cells (Sup. Fig. 1D). The percentage of γδ cells, determined in 8 week old goats in an additional staining using the WC1 γδ T-cell antibody, was below 10% in the majority of samples analysed (Sup. Fig. 2A). The presence of $WC1^+$ γδ cells within $CD4^+$ cells was almost negligible, $CD8^+$ lymphocytes contained up to 10% γδ lymphocytes (Sup. Fig. 2B and Sup. Fig. 2C).

Due to the use of magnetic-bead enrichment in humerus BM, we were not able to compare the percentages of $CD4^+$ and $CD8^+$ cells between BM and the other organs, however CD4/CD8 ratios could be compared.

2.6. Cell sorting

$CD4^+$ and $CD8^+$ cells ($7AAD^-$ and $CD14^-$) from pre-scapular LN and blood were sorted (purity > 90%) on a FACS Aria III cell sorter (BD Biosciences) using FACS Diva software (BD Biosciences). Granulocytes were isolated by cell sorting from lysed whole blood based on forward/side scatter characteristics.

2.7. DNA isolation

Genomic DNA was isolated from $CD4^+$ and $CD8^+$ cells sorted from blood and LN, total thymocytes, and granulocytes using the Reliaprep Blood gDNA Miniprep System (Promega, Madison, WI, USA) and stored at −20 °C before processing for gas chromatography/mass spectrometry (GC/MS).

2.8. Measurement of ^2H_2O enrichment in body water and DNA

Deuterium enrichment in urine, plasma and DNA was analysed by GC/MS using an Agilent 5973/6890 GC/MS system (Agilent Technologies). Urine and plasma were derivatized to acetylene (C_2H_2, M = 26) as previously described (Westera et al., 2013b). The derivative was injected into the GC/MS equipped with a PoraPLOT Q 25 × 0.32 column (Varian), and measured in SIM mode monitoring ions m/z 26 (M+0) and m/z 27 (M+1). DNA was hydrolysed to deoxy-ribonucleotides and derivatized to penta-fluoro-triacetate (PFTA, M = 435) (Westera et al., 2013b). The derivative was injected into the GC/MS equipped with a DB-17 column (Agilent Technologies) and measured in SIM mode monitoring ions m/z 435 (M+0), and m/z 436 (M+1). Because urine was used for neonatal kids and plasma for adult goats, based on paired plasma and urine enrichments from 19 neonatal kids we confirmed that urine and plasma have very similar enrichment levels (Ackermans et al., 2001) (Sup. Fig. 3).

2.9. Mathematical modelling of urine, plasma and DNA enrichment data

To control for changing levels of ^2H in body water over the course of the experiment, a simple label enrichment/decay curve was fitted to ^2H enrichment in plasma and urine:

during label intake ($t \leq \tau$): $S(t) = f(1 - e^{-\delta t})$
after label intake ($t > \tau$): $S(t) = [f(1 - e^{-\delta \tau})]e^{-\delta(t-\tau)}$

as described previously (Vrisekoop et al., 2008) (with minor modification because there was no initial boost of label), where *S(t)* represents the fraction of ^2H_2O in plasma or urine at time t (in days), f is the fraction of ^2H_2O in the drinking water or milk, labelling was stopped at $t = \tau$ days, and δ represents the turnover rate of body water per day. The best fit for *S(t)* was used in the labelling equations for the different cell populations (see below). Up- and down-labelling of the granulocyte population was analysed as previously described (Vrisekoop et al., 2008), to estimate the maximum level of label intake that cells could possibly attain (Sup. Fig. 4, Sup. Fig. 5, and Sup. Table 1). The label enrichment data of all cell subsets were subsequently scaled by the granulocyte asymptote (Vrisekoop et al., 2008).

Although cell numbers in most compartments of neonatal goats did not increase significantly with age, the higher cell numbers in adult goats clearly showed that $CD4^+$ and $CD8^+$ T-cell numbers in neonatal kids were not yet in steady state. To study T-cell dynamics in neonatal kids, we therefore used a slightly altered version of our previously published mathematical model (Westera et al., 2013a) by releasing the assumption that cell numbers are in steady state. In this model, we assume that $CD4^+$ and $CD8^+$ T-cells are produced by a source σ (cells/day), proliferate at a rate p (per day) and are lost at a rate d (per day):

$$\frac{dN}{dt} = \sigma + pN - dN$$

The fraction of labelled DNA (l) is hence described by the following differential equation:

$$\frac{dl}{dt} = \left(\frac{\sigma}{N} + p\right)(cS(t) - l)$$

where c is the amplification factor accounting for the fact that multiple hydrogen atoms can be replaced by deuterium (Vrisekoop et al., 2008) and *S(t)* represents the deuterium enrichment in the body water, as described above. The average turnover rate (i.e. the *per capita* production rate, ρ) that was estimated is the joint term $\left(\frac{\sigma}{N} + p\right)$, where the number of cells N is considered (relatively) constant but not necessarily in steady state.

For adult goats, we assumed cell numbers to be in steady state, and used the multi-exponential model allowing for hetereogeneity between cells of the same population that we have used before (Westera et al., 2013a).

Because all enrichment data were expressed as fractions, labelling data were arcsin(sqrt) transformed before the mathematical model was fitted to the data. For the adult goats, we followed a stepwise selection procedure to determine the number of kinetically different subpopulations to include in the model, adding a new kinetically different subpopulation into the model until the average turnover rate no longer changed (Westera et al., 2013a). For populations that appeared to behave kinetically homogeneously, the fitting procedure set the contribution of the extra subpopulation(s) to zero. The labelling curve of $CD4^+$ cells from blood of adult animals was significantly better described by a model including two kinetically different subpopulations; the other populations required only one subpopulation.

2.10. Data analysis

Differences between groups were assessed using Wilcoxon signed-rank test in Prism (GraphPad software Inc, La Jolla, CA, USA). Differences with a p-value < 0.05 were considered significant. Characteristics of young and adult animals were compared by performing Wilcoxon signed-rank test between the pooled data of neonatal goats and the pooled data from 2 to 6 year old adult goats. Linear regression analysis was performed using Prism 6 (GraphPad software Inc, La Jolla, CA, USA). Regression lines with a significant slope ($y = ax + b$, where a was significantly different from 0) are represented by solid lines, whereas dashed lines represent regression lines with a slope that did not significantly deviate from 0.

3. Results

3.1. Changes in weight and leukocyte numbers of primary and secondary lymphoid organs over time

Goats grew rapidly during the first 5 months of life and gained on average 1.2 kg body weight per week. The weight of adult animals differed substantially between animals, between ∼50 and 70 kg (Fig. 1A). The weight gain of the animals recorded in this study was in line with previously reported weight curves (Malone et al., 2013).

First, we studied how the weight and the leukocyte cellularity of primary and secondary lymphoid organs changed over time. The weight of the thymus in neonatal kids increased significantly between the foetal stage and 5 months of age; in fact the thymus was the only organ whose weight grew more rapidly than that of the animal, as even the ratio of thymus weight over total body weight increased with age (p-value = 0.009) (Fig. 1B). During the prenatal period the thymus was already populated with lymphocytes, however the foetal thymus contained fewer leukocytes than that of neonatal kids (i.e. animals of 1–5 months of age, Fig. 1B). In adult animals, thymus weight was much

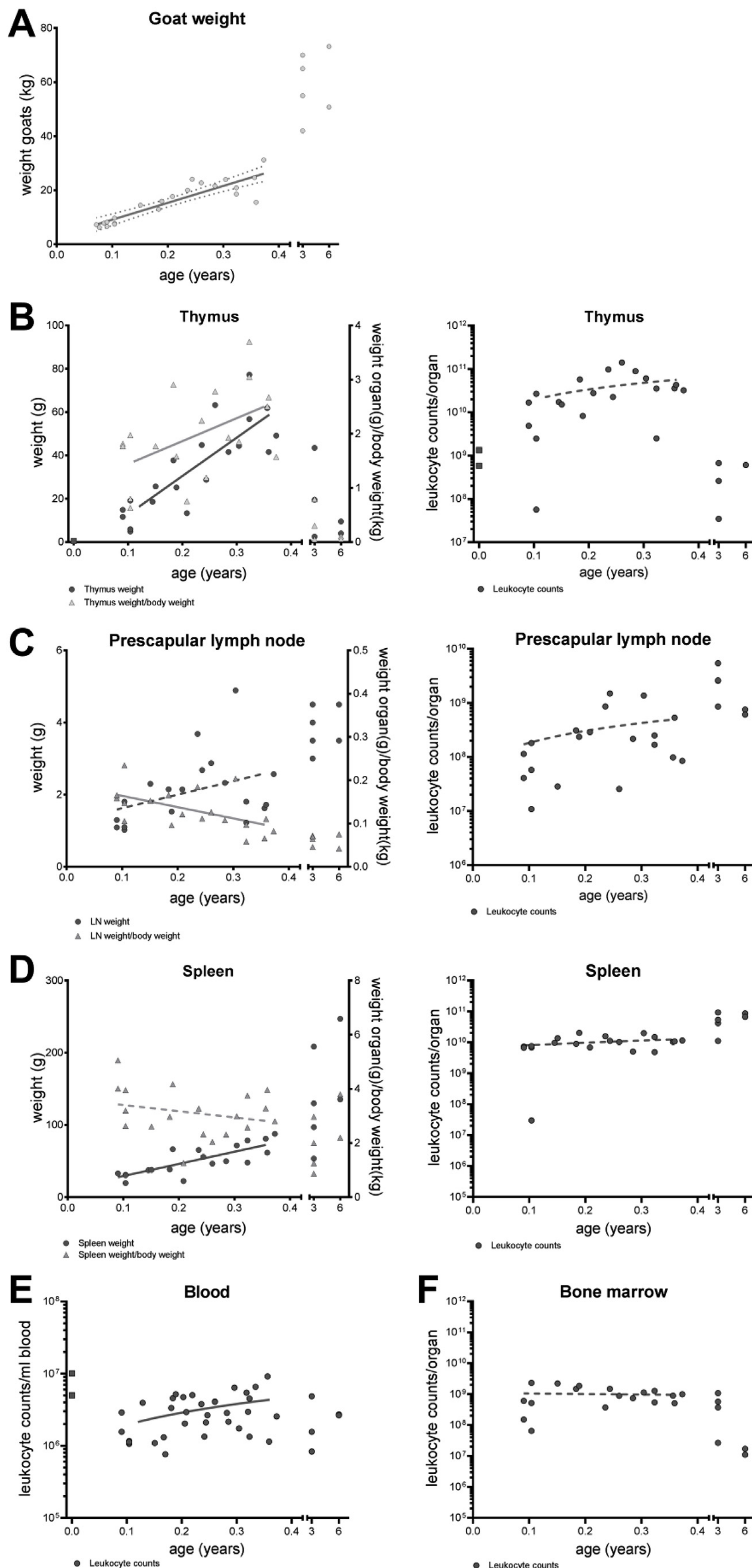

Fig. 1. Changes in weight and total leukocyte counts per organ over time in goats. Goat weight (in kg) increased linearly with age (p-value < 0.0001) **(A)**. Left panels show the changes in organ weight (in grams, denoted by (○), see left axes), and in the weight of the organ (in grams) divided by the animal body weight (in kg) (denoted by (Δ), see right axes) over time, for thymus **(B)**, pre-scapular LN **(C)**, and spleen **(D)**. Right panels show the corresponding leukocyte counts per organ (plotted on a logarithmic scale), which were obtained using a Cell-DYN Emerald Haematology counting system. Panel **(E)** gives the total leukocyte counts per ml of blood and panel **(F)** the total leukocyte counts per bone. Square symbols (□) correspond to data from 2 foetuses. Regression lines with a significant slope are represented by solid lines, dashed lines represent non-significant slopes. Thymus weight and thymus weight corrected for body weight significantly increased from 1 to 4.5 month of age (right panel **(B)**, p-value < 0.0001 and p-value = 0.026, respectively). Spleen weight also increased significantly between 1 and 4.5 months of age (right panel **(D)**, p-value < 0.0001). In contrast, prescapular LN weight corrected for body weight decreased over time (right panel **(C)**, p-value = 0.014). During the study period, both in neonatal kids (between 1 and 4.5 months of age) and in adult animals (2–3 years of age), total leukocyte numbers per thymus, prescapular LN, spleen, and BM did not change significantly (right panel **(B)**, **(C)**, **(D)** and **(F)**). Only in neonatal kids did we observed a significant increase in leukocyte counts per ml of blood (panel **(E)**, p-value = 0.020) over the 5 months study period.

lower than in 5 months old goats and thymocyte counts were significantly lower than in neonatal kids (p-value = 0.034). In contrast to what was observed for the thymus, the weight and cellularity of LN and spleen were higher in adult animals compared to neonatal kids (p-value = 0.038 for LN weight and p-value = 0.057 for LN cellularity; p-value = 0.0087 for spleen weight and cellularity) (Fig. 1C and Fig. 1D). Foetal cord blood contained more leukocytes per ml than blood from 1 month old new-borns. After this initial drop in blood leukocyte numbers, leukocytes per ml of blood increased significantly between the 1st and 5th month of life and finally stabilized at adult age (Fig. 1E). For the BM, the number of leukocytes per bone was stable during the neonatal period, but tended to be lower in 6 year old animals (Fig. 1F).

Despite the rapid growth of the animals between 2 weeks and 5 months of age, total leukocyte numbers per organ did not significantly increase in the main lymphoid organs during that period of time. Nevertheless, total leukocyte numbers per organ were significantly higher in adult animals compared to neonatal kids, most likely due to the difference in organ size.

3.2. Alterations in the T-cell compartment during development and adulthood

In a large number of livestock species including ruminants, pigs and poultry, γδ T-cells are present at high percentages in circulation (Holderness et al., 2013). However, a study in goats previously showed that the frequency of circulating γδ T-cells in goats is rather small, both in young and adult animals (Caro et al., 1998), suggesting that goats are not a γδ high species. Here, we first determined the percentage of γδ lymphocytes in goats in different organs at 8 weeks of age. In line with previous reports (Caro et al., 1998), we found that the frequency of γδ T-cells in goats was low. Blood and spleen contained the highest percentages of γδ-lymphocytes. The fraction of $WC1^+$ γδ T-cells within lymphocytes was approximately 10% for blood and spleen, and below 5% for LN, gut, thymus and BM (Sup. Fig. 2A). The percentage of $WC1^+$ γδ T-cells within lymphocytes in neonatal goats was significantly lower than in young cattle, in which γδ T-cells can comprise up to 60% of blood lymphocytes (Mackay and Hein, 1989).

Given the low frequency of γδ T-cells we focussed our analysis on αβ T-cell compartment in goats. We characterized the $CD4^+$ and $CD8^+$ cells within $CD14^-$ lymphocytes. We have recently shown that at a transcriptional level, $CD4^+$ and $CD8^+$ $CD14^-$ cells in goats show high expression of the CD3E transcript, despite not having been sorted based on CD3 (Baliu-Piqué et al., 2018). Based on this finding, we assume that the sorted $CD4^+$ and $CD8^+$ $CD14^-$ cell populations studied here also expressed CD3, hence we refer to them as T-cells.

During the neonatal period, $CD4^+$ cells were the predominant T-cell subset in blood, thymus and LN, while $CD8^+$ cells were the predominant T-cell subset in spleen and BM (Fig. 2, Table 1). The T-cell subset distribution was relatively stable during the first 5 months of life in all organs analysed except for the pre-scapular LN, where the percentage of $CD4^+$ T-lymphocytes decreased from approximately 50%–30% between 1 and 5 months of age (Fig. 2B, Table 1). The T-cell composition of the thymus, LN, spleen and BM were quite similar in adults and neonatal kids, while the composition of blood changed from a median CD4/CD8 ratio of 2.5 towards a ratio below 1 (Fig. 2, Table 1). This was due to a decrease in the percentage of $CD4^+$ T-cells (p-value = 0.0048) and an increase in the percentage of $CD8^+$ T-cells (p-value = 0.0027) between neonates and adult goats (Fig. 2D, Table 1).

The foetal thymus contained similar percentages of $CD4^+$ and $CD8^+$ single positive (SP) cells as that of neonatal kids and adult animals (Table 1). In contrast, the percentages of $CD4^+$ and $CD8^+$ T-cells in foetal cord blood were below 4% and 2%, respectively (Fig. 2A, Table 1), much lower than in blood from neonatal kids and adult animals (Table 1).

3.3. $CD4^+$ and $CD8^+$ T-cell dynamics in neonatal kids and adult animals

To study whether the changes in cell density and composition of the T-cell compartment with age were accompanied by changes in the dynamics of T-cells, we used *in vivo* deuterium labelling in neonatal kids and adult animals to quantify the turnover rate of $CD4^+$ and $CD8^+$ T-cells. Two groups of neonatal kids, 12 animals each, and 34 adult animals received ^2H_2O for 4 weeks and were sacrificed at different time points during the labelling and de-labelling period, such that a cross-sectional up- and down-labelling curve of deuterium enrichment could be constructed. Using mathematical modelling (see material and methods), we estimated the average turnover rate (ρ) of $CD4^+$ and $CD8^+$ T-cells, i.e. the fraction of cells replaced by new cells per day.

The deuterium enrichment levels in $CD4^+$ and $CD8^+$ T-cells in the 2 groups of neonatal kids were very similar, and both T-cell subsets reached higher deuterium enrichment levels in neonatal kids than in adult goats (Fig. 3A and Fig. 3B). The best fits of the model to the experimental data (Fig. 3A and Fig. 3B) and their corresponding parameters revealed that the average turnover rates of $CD4^+$ and $CD8^+$ T-cells in neonatal kids were ~10-fold faster than in adult animals. The estimated parameters should, however, be interpreted with care as the confidence intervals are rather large, especially for adult animals. Based on the data from blood, the estimated average turnover rate of $CD4^+$ T-cells was 0.050 per day for neonatal kids of group-1, 0.070 per day for neonatal kids of group-2, and 0.011 per day for adult goats. For $CD8^+$ T-cells, we estimated an average turnover rate of 0.114 per day for neonatal kids of group-1, 0.207 per day for neonatal kids of group-2, and 0.015 per day for adult animals (Fig. 3C). Based on data from the LN, the estimated average turnover rate of $CD4^+$ T-cells was 0.058 per day for neonatal kids of group-1, 0.043 per day for neonatal kids of group-2, and 0.005 per day for adult animals. For $CD8^+$ T-cells isolated from the LN, we estimated an average turnover rate of 0.028 per day for neonatal kids of group-1, 0.109 per day for neonatal kids of group-2 (Fig. 3C), and 0.003 per day for adult animals (for 95% confidence intervals see Sup. Table 2).

In summary, *in vivo* deuterium labelling revealed that $CD4^+$ and $CD8^+$ T-cells of neonatal kids have a 5- to 10-fold higher average turnover rate than $CD4^+$ and $CD8^+$ T-cells of adult animals.

4. Discussion

The presence of a dedicated organ for T-cell development, the

Table 1
Characterization of the T-cell compartment of goats. Fraction of $CD4^+$ and $CD8^+$ T-cells as percentage of total single lymphocytes, and the CD4/CD8 ratio are shown (mean and SD). The reported percentages of $CD4^+$ and $CD8^+$ T-cells of BM from neonatal kids were based on BM samples from six animals; these were the only animals for which BM samples were analysed by flow cytometry before magnetic bead enrichment.

Organ	Thymus			Lymph node		Spleen		Blood			Bone marrow	
Age group	Foetus	Neonate	Adult	Neonate	Adult	Neonate	Adult	Foetus	Neonate	Adult	Neonate	Adult
% $CD4^+$	7.2 (0.1)	10.4 (13.7)	13.7 (10.9)	37.5 (12.5)	33.4 (12.1)	10.7 (4.5)	6.7 (2.74)	2.3 (0.9)	25.5 (7.7)	17.7 (6.5)	0.45 (0.2)	1.1 (0.8)
% $CD8^+$	2.6 (0.2)	4.3 (5.3)	8.5 (12.5)	10.5 (3.3)	16.6 (4.4)	18.9 (6.9)	19.1 (12.5)	0.8 (0.4)	9.6 (3.2)	25.4 (8.4)	0.82 (0.46)	5.4 (2.5)
CD4/CD8	2.8 (0.3)	3.5 (2.3)	5.9 (4.5)	3.7 (1.1)	2.1 (0.9)	0.7 (0.4)	0.9 (0.9)	3.2 (0.3)	2.7 (0.9)	0.8 (0.5)	0.6 (0.3)	0.2 (0.1)

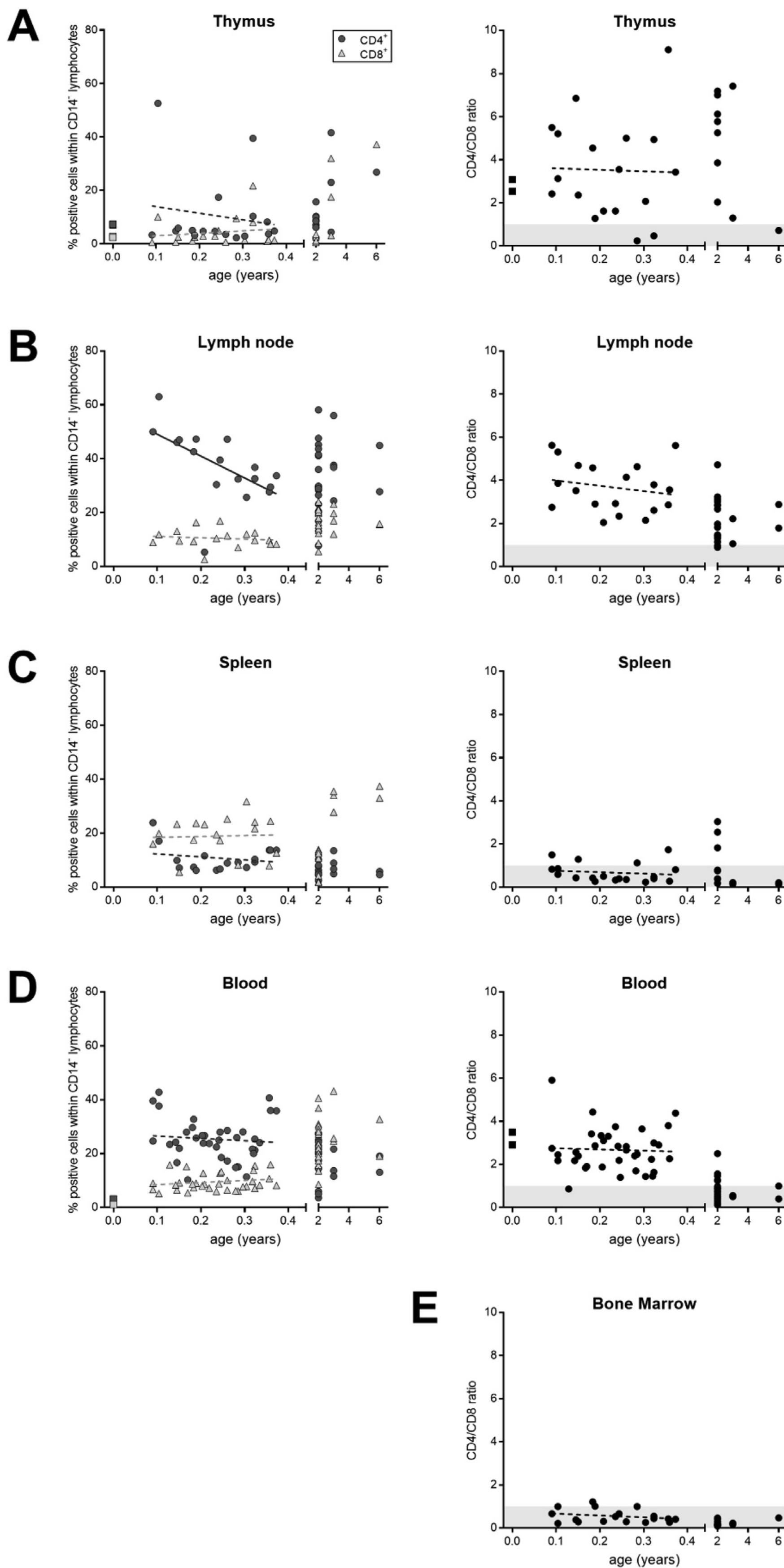

Fig. 2. Contribution of $CD4^+$ and $CD8^+$ T-cells. Left panels show the percentage of CD4 (○) and CD8 (Δ) single positive cells within the $CD14^-$ lymphocytes for thymus **(A)**, pre-scapular LN **(B)**, spleen **(C)** and blood **(D)**. Right panels present the ratio of CD4/CD8 over time for the different organs and **(E) for the BM**. Square symbols (□) correspond to data from 2 foetuses. Regression lines with a significant slope are represented by solid lines, dashed lines represent non-significant slopes. Grey area corresponds to a CD4/CD8 ratio below 1. In neonatal kids (between 1 and 4.5 months of age) the frequencies of $CD4^+$ and $CD8^+$ cells were relatively stable in the thymus, spleen, and blood (left panels **(A)**, **(C)** and **(D)**. The percentage of $CD4^+$ T-cells in the LN decreased significantly between 1 and 5 month of age (left panel **(B)**, p-value = 0.017).

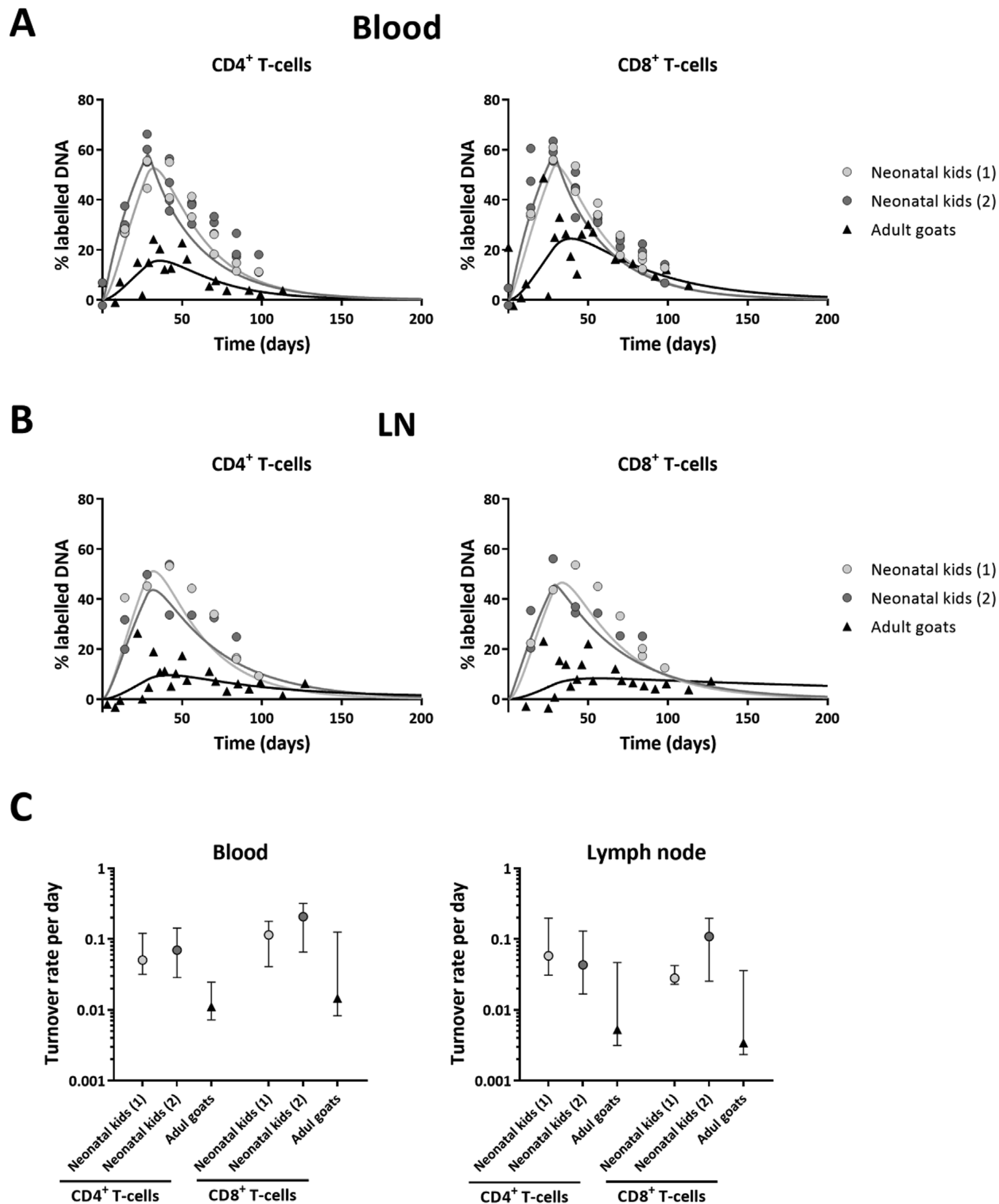

Fig. 3. Dynamics of CD4$^+$ and CD8$^+$ T-cells from blood and LN in neonatal and adult goats. Best fits of the mathematical model to the level of deuterium enrichment measured in the DNA of total CD4$^+$ and CD8$^+$ T-cells from blood **(A)** and LN **(B)** of neonatal kids (group 1 and group 2) and adult goats. Label enrichment in the DNA was scaled between 0 and 100% by dividing by the estimated maximum deuterium enrichment of granulocytes (See material and methods). Estimated turnover rates (plotted on a logarithmic scale), i.e. *per capita* production rates, of CD4$^+$ and CD8$^+$ T-cells from blood and LN of neonatal kids (group 1 and group 2) and adult goats, and their respective 95% confidence limits are given in panel **(C)**.

thymus, extends to the most ancient vertebrates (Ge and Zhao, 2013). The thymus is needed to develop immunity even before birth, and it is of extreme importance during the early postnatal period for *de novo* generation of T-cells. Most studies on thymic function have been performed in mice and data on thymic growth, involution and export in other mammals are very scarce. Mice have a relatively short gestation period of ~20 days, in contrast to humans, goats and sheep which have much longer gestational periods. During fetal life, lymphoid tissues, including the thymus, of both humans and ruminants are already populated with T-cells, and these cells are also found in the periphery prior to birth (Antony et al., 2011; Baldwin and Telfer, 2015; Cunningham et al., 2001). In line with this, we found that the foetal thymus in goats was already populated with CD4$^+$ and CD8$^+$ single positive lymphocytes and that CD4$^+$ and CD8$^+$ T-cells were present in foetal cord blood. When the first double positive and single positive CD4$^+$ and CD8$^+$ lymphocytes appear in the thymus and how their development in the thymus compares to that of WC1$^+$ γδ T-cells remains to be studied. Based on thymic mass and cellularity, our data suggest that thymic involution in goats is minimal during the first 5 months after birth, but significant later in life. Thymic mass and

cellularity are of course no direct measures of thymic output, but data from sheep have suggested that daily thymic output is linearly related to thymocyte numbers (Cunningham et al., 2001). If this is also the case in goats, our data suggest that daily thymic output is relatively constant in neonatal kids and significantly reduced in adult goats. Similarly, in humans, involution of the thymus is thought to begin during or soon after the first year of life (Steinmann, 1986; Steinmann et al., 1985). In the BM, a very similar process was observed. Leukocyte numbers per bone remained stable during the first 5 months of life, but had decreased in adult animals, likely due to accumulation of adipose tissue in BM. This phenomenon, called fatty involution of the BM, has also been described in humans (Justesen et al., 2001) as well as in other species (Bigelow and Tavassoli, 1984).

Despite the rapid growth of the goats in the first half a year of life, the weight of their prescapular LN was relatively constant from birth to the first 5 months of life, suggesting that the LN was fully or close to fully grown and developed at birth. In contrast to the early development of the LN, the spleen significantly increased in weight during the first 5 months of life. Total leukocyte numbers per prescapular LN and spleen did not increase significantly between 1 and 5 months of age, probably because of the relatively short duration of the study and the uncertainty inherent to measuring absolute cell numbers. Leukocyte numbers in the prescapular LN and the spleen, indeed, significantly increased between neonatal kids and adult goats. For blood, we observed a significant increase in the leukocyte numbers per ml of blood from 1 to 5 months of age. In line with previous observations in humans (van Gent et al., 2009), both leukocyte counts per ml of blood and the ratio of CD4/CD8 cells decreased in the long term between neonatal kids and adult animals.

In the current study, we focussed on the composition and dynamics of αβ CD4$^+$ and CD8$^+$ T-cells in various lymphoid compartments and peripheral blood during development and adulthood. We observed that, in blood, CD4$^+$ T-cells were the predominant subset during the neonatal period, while CD8$^+$ T-cells predominated in adults. We cannot exclude the possibility that changes in the size of the γδ T-, NK- and B-cell populations could account, to a certain extent, for the observed changes in the percentages of CD4$^+$ and CD8$^+$ T-cells. It has been previously shown that, indeed, there are some changes in percentages of IgM$^+$ and γδ T-cells over time (Caro et al., 1998), which suggests that these populations may play a role. We determined the percentage of γδ-lymphocytes in 8 week old goats. In agreement with Caro *et al.* Caro et al. (1998), we found that between 5 and 10% of the total lymphocytes were WC1$^+$ γδ T-cells, much lower than in cattle (Baldwin and Telfer, 2015; Mackay and Hein, 1989). This suggest that γδ T-cells may play a smaller role in immune protection in goats than in cattle. It would interesting to obtain insights in the changes of B-, γδ T- and NK-cells during development and adulthood to further address this issue, which is now possible thanks to the steady increase in the number of available reagents for goats. Furthermore, studies on the function of CD4$^+$ and CD8$^+$ T-cells from birth to adults in goats and other ruminants would be relevant to determine whether, as observed in human and mice, their functional activity is altered early in life. In line with this, a previous study has shown that neonatal goats have a stronger TH1 cytokine response to TLR ligands than adult animals (Tourais-Esteves et al., 2008).

In vivo deuterium labelling studies are typically restricted to adults, i.e. experimental limitations due to the small size of neonatal mice and ethical concerns in humans. Goats are a good alternative model to study the human pre and early postnatal period. Large volumes of blood and tissues can be obtained both from neonatal and adult animals, from which enough CD4$^+$ and CD8$^+$ T-cell can be isolated for all types of analysis. Secondly, as previously mentioned they are similar in immunological development. *In vivo* ^2H_2O labelling in goats revealed that CD4$^+$ and CD8$^+$ T-cell turnover rates are approximately 5- to 10-fold faster in 5-month old neonatal kids than in adult goats (2 years of age), suggesting that the T-cell pool in neonates is more dynamic than in adults. Based on deuterium labelling data alone one cannot distinguish which part of the enrichment was obtained by proliferation in the thymus and which by cell division in the periphery. Indeed, the estimated turnover rate (ρ) from our mathematical model is defined as $\left(\frac{\sigma}{N} + p\right)$ which is hence a *per capita* production rate, in which σ is the daily thymic output, N is the total cell number and p is the peripheral division rate. Since total leukocyte numbers (N) were about 10-fold lower in neonatal kids and the thymus size was 100-fold higher than in adult goats, the increased *per capita* production rates in neonatal kids could be due to a larger daily thymic output (σ) feeding into a smaller total pool size (N), in the absence of increased peripheral T-cell division rates (p). Thus, the increased *per capita* production rates in young goats should not be interpreted as evidence for increased T-cell *proliferation* rates during early development, as has been previously suggested in mice (Min et al., 2003).

It is important to realize that the composition of the CD4$^+$ and CD8$^+$ T-cell pools may have been different between neonates and adults. In humans, naive T-cells are the predominant population (up to 95%) of CD4$^+$ and CD8$^+$ T-cells in the blood, spleen, lymph nodes and colon during the first 2 years of life, while in young adults they are present at much lower frequencies (Thome et al., 2016). It is likely that neonatal and adult goats, like humans, have a different T-cell subset distribution throughout the body. Although we cannot formally exclude the possibility that the differences observed in T-cell dynamics between neonatal and adult animals were merely due to differences in the naive and memory composition of their T-cell pools, we think this would be highly unlikely because one would expect neonatal kids to have higher percentages of naive T-cells, which typically have slower turnover rates than memory T-cells (Vrisekoop et al., 2008; Westera et al., 2015). The surface molecules CCR7 (CC chemokine receptor 7) and CD62L (Selectin-L) have recently been proposed to distinguish the naive and memory T-cell populations in goats (Baliu-Piqué et al., 2018), which would enable the study of the dynamics of these cell populations in more detail in the future.

Stable isotope labelling studies in humans are typically restricted to blood. In our study, we had the opportunity to simultaneously compare the deuterium enrichment levels in blood and LN. We consistently found the enrichment levels to be slightly lower in LN compared to blood for CD4$^+$ and CD8$^+$ cells both in neonatal and adult goats (Fig. 3A and Fig. 3B), although these differences did not reach statistical significance. Our results are in line with previous observations in humans. Kovacs *et al.* determined the level of deuterium incorporation of human LN CD4$^+$ and CD8$^+$ T-cells obtained 3 months after labelling from LN biopsies of 2 individuals. In line with our results, they observed that the labelling of T-cells derived from the LN was slightly lower compared to that of T-cells taken from peripheral blood (Kovacs et al., 2005). We and others have observed that T-cells isolated from the LN produce less cytokines compared to their counterparts isolated from blood after *in vitro* stimulation (Baliu-Piqué et al., 2018; Hill et al., 2014; Sopp and Howard, 2001). Taken together, these findings suggest that blood is enriched for T-cells that are more active than those in LN, which might reflect an effect of the different “environments” of the cells, differences in the frequency of naive and memory T-cells between blood and LN (Minang et al., 2010), or a bias of T-cells to go into the circulation after their activation in the LN.

5. Conclusion

Our data suggest that the composition of the goat neonatal αβ T-cell pool differs from that of adult animals both quantitatively and qualitatively. Quantitatively, in comparison to adults, neonates contain lower numbers of splenic leukocytes and fractions of T-cells in peripheral lymphoid tissues and blood. Qualitatively, neonatal T-cells are very dynamic with average turnover rates that are 5- to 10- fold faster than those in adults. We found clear similarities in the development of

T-cells in goats and humans, including a decreasing CD4/CD8 ratio, thymic involution, and decreasing leukocyte cellularity in BM with age. Future studies characterizing functional differences between the T-cell compartment of neonates and adults are needed to study whether and how these changes are related to the increased disease susceptibility and sub-optimal vaccine response of neonates. Such studies could take advantage of goats and other ruminants as experimental models for developmental immunology.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2018.12.004.

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