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The Immune Status of Bovine Somatic Clones

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Abstract

Agronomical applications of cloned livestock produced by somatic cell nuclear transfer (SCNT) have been authorized in the United States and the European Food Safety Authority published that there was no evidence of risks associated with the use of cloned animal in the breeding industry. Both assessments, however, underlined that complementary data are needed to update their conclusions. SCNT is associated with a high incidence of perinatal losses. After birth, cloned cattle appear to possibly present subtle immune defects, requiring extensive studies to be properly evidenced. Twenty-five cloned Holstein heifers from five distinct genotypes and their contemporary age- and sex-matched controls were compared. An extensive survey of leukocyte subsets was performed and the humoral and T-cell immune responses to exogenous antigens were studied. Cloned cattle presented a normal representation of leukocyte subsets. Functional immunity was not modified in cloned heifers, as they were able to raise an antibody response and to develop B and T cell-specific responses against the model antigen OVA (ovalbumin) and against a rotavirus vaccine as in controls. Thus, this extensive analysis supports previous data suggesting that cloned cattle have a normal immunity.

Introduction

Agronomical applications of cloned livestock produced by somatic cell nuclear transfer (SCNT) have been authorized in the United States based on a thorough study of scientific evidence available internationally on the health and quality of products obtained from animal clones (Food and Drug Administration, 2008). Similar decisions have been published by other national food safety authorities such as New Zealand. In 2008, the European Food Safety Authority (EFSA) published that there was no evidence of risks associated with the use of cloned animal in the breeding industry (Barlow et al., 2008). However, both assessments underlined that continuing data are needed to update their conclusions. In particular, EFSA clearly stated that "the low number of animals and the few assays carried out do not allow precise measurement of the impact of cloning on the immune functions of the cloned animals. Such an impact, if present, could modify the carrier state of the cloned animals with respect to infectious agents of animal and human health concern." It is then recommended that further investigations should be conducted to determine "the causes of pathologies and mortality observed in clones during the gestational and postnatal periods and those observed at a lower frequency in adulthood."

SCNT is associated with a high incidence of fetal losses in all pregnancy stages in cattle. In late gestation, these losses are related to the occurrence of placental abnormalities (placentomegaly, hydrops) (Constant et al., 2006; Heyman et al., 2002; Miglino et al., 2007) and abnormal placental gene expression (Everts et al., 2008). In the early pregnancy, a delay in implantation has been described with defaults in placental vascularisation (Hill et al., 2000). Overexpression of major histocompatibility complex class I (MHC-I) molecules at the trophoblastic surface and high lymphocytic ($CD3^+$ T) infiltration in the endometrium have been suggested as possible causes for the early fetal losses in clones (Hill et al., 2002). However, these observations were presumably genotype-dependent, as these results could not be repeated in a similar study conducted with other genotypes (Chavatte-Palmer et al., 2007). In any case, these results raise questions about a possible perturbation of immunity in clones.

After birth, cloned cattle appear to present subtle immune defects. In the neonatal period, thymic atrophy has been

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reported in some cloned calves before 6 months of age (Kubo, 2002; Renard et al., 1999), together with an increased incidence of infections in young animals (Chavatte-Palmer et al., 2004) The mortality in adult cloned cows has been reported to be higher than in control animals (8% mortality per year in clones before 4 years of age vs. 2–3% in controls) with a high incidence of musculoskeletal abnormalities causing chronic lameness in milking cows (Wells et al., 2004). However, in adult bovine clones, Lanza et al. (2001) reported normal proportions of B lymphocytes and of MHC-I$^+$, MHC-II$^+$, CD4$^+$, CD8$^+$, CD45$^+$ leukocytes. Moreover, peripheral blood mononuclear cells (PBMCs) from clones showed a normal proliferative response to phytohemagglutinin (PHA) (Lanza et al., 2001). In another study, no differences were observed in the proportions of granulocytes, monocytes, B cells, total T cells between lactating Holstein clones and their noncloned comparators (Tanaka et al., 2006). Interestingly, a difference was detected in the proportions of $\gamma\delta$ T lymphocytes in clones in early lactation compared to controls, but these results may have resulted from differences in milk production. Finally, when adult bulls were grafted with skin tissue from an unrelated animal, the cloned bulls took longer to reject the graft compared to the control, suggesting that they may have been more immunotolerant (Theoret et al., 2006).

In mice, antibody production following injection of live bacteria was significantly reduced in clones relative to age- and genotype-matched controls (Ogonuki et al., 2002). The cloned mice died prematurely, and the deficits in immune functions were believed to be the cause of the pneumonia observed at postmortem examination (Ogura et al., 2002).

In piglets, the cortisol, interleukin 6, and tumor necrosis factor responses to a lipopolysaccharide injection were lower in clones compared to controls. However, the cytokine responses were markedly different between two groups of clones generated from two genetically identical donor cell lines. These results suggested that the acute phase response was altered in cloned piglets, with differences related to the cell line of origin (Carroll et al., 2005).

Overall these data underline that the cloning technology may generate subtle differences in the immune status that may require extensive studies to be properly evidenced. The work presented here is part of a larger study on the health and quality of products derived from cloned cattle which results have been summarized elsewhere (Heyman et al., 2007a, 2007b; Jurie et al., 2008). Our goal was to further analyze the immune status of cloned cattle by performing an extensive description of the leukocyte subsets and an evaluation of the humoral and T-cell immune responses on a consistent number of cloned cattle from several distinct genotypes (25 cloned cattle from five distinct genotypes). We focused our study on cloned cattle that have passed the neonatal period, mostly after 2 months of age, because at this stage the period of heavy neonatal loss is over (Chavatte-Palmer et al., 2002, et al. 2004; Wells et al. 2004; Wells 2005), although we also examined a few healthy looking neonatal calves. Regarding the leukocytes subsets involved in acquired immunity, we conducted a thorough phenotypic description of the B and T cells subset represented. Ruminants present a large B-cell subset in their blood designated as the B1-like subset (CD5$^+$ CD11b$^+$ B cells) (Chevallier et al., 1998). B1 cells are considered in many species to mediate "natural" immunity based on their ability to produce large amounts of multireactive IgM, IgG3, and IgA (Tarakhovsky, 1997). Their representation is often altered in immune insufficiency conditions (Raveche, 1990). In addition, in cattle, B1-like cells are the specific target of bovine leukemia virus and of theileria annulata (Moreau et al., 1999) and they appear prone to dysregulation during infections (Buza et al., 1997). Among the classically studied CD8$^+$ and CD4$^+$ α/β T-cell populations, some subsets are endowed with specific functions in many species that are also conserved in the bovine species: (1) the CD4$^+$ CD25$^+$ T cells that show regulatory properties (Seo et al., 2007); (2) the CD4$^+$ CD45RO$^+$, and CD8$^+$ CD45RO$^+$ memory subsets (Bembridge et al., 1995); (3) the CD4$^+$ MHC2$^+$ that correspond to activated T cells (Holling et al., 2004). Finally, we also evaluated the representation of the inflammatory γ/δ T subset [workshop cluster 1+ (WC1$^+$)] cells (Meissner et al., 2003) as it was found altered in a previous study on cloned cows at the beginning of lactation (Tanaka et al. 2006). In cattle as in humans and mice, g/d T cells are involved in innate immunity by providing a rapid cytokine response to poorly defined agonists such as tanins (Jutila et al., 2008).

Our results show that cloned cattle presented a normal representation of leukocyte subsets. Furthermore, their immune responses to vaccines against ovalbumine or rotavirus were similar to control cattle, suggesting that cloning does not affect immunity in cattle, at least in the postneonatal period.

Animals, Materials and Methods

Animals

Altogether, 50 Holstein heifers were involved in the present experiment. Twenty-five of them were clones obtained by SCNT [SCNT cows, genotypes A ($n=8$), C ($n=3$), D ($n=8$), F ($n=5$), G ($n=1$)] and 25 animals were produced by artificial insemination and served as controls. All animals used were female, age-matched pairs (one clone and one control). The experiments first started with the immunology and immunophenotyping. At that time, three clone heifers and their three contemporary age-matched controls were less than 2 months of age. The same pairs were subsequently used at 5–6 months of age to test the immune response to antigens.

For hematological analysis and immunophenotyping of PBMCs subsets, 30 clinically healthy animals (15 clones and 15 controls) were used, including six calves (three clones and three controls) under 1 month of age. Blood was drawn simultaneously for hematology and immunophenotyping on a set day, and a second sample was drawn within a week of the first sampling for a replicate immunophenotyping. Mean age was 13.2 months (range 1 week to 60 months).

For the vaccination experiments, 26 animals were used (13 clones and 13 controls, including the six young calves previously used for hematology and immunophenotyping). The mean age was 10.3 ± 3.2 month (range = 5–17 months). Twelve heifers were used for a rotavirus vaccination protocol and 14 were used to test their response to ovalbumin vaccination.

Production of cloned animals

Skin biopsies were taken from the ear of five different Holstein donor cows to generate fibroblasts cell lines that were cryopreserved in liquid nitrogen. These cells were used

for SCNT procedure according to the method previously described in our lab (Vignon et al., 1998). Briefly, recipient oocytes were matured *in vitro*. Bovine ovaries were collected at the abattoir, washed several times with fresh saline, and transported in sterile PBS at 33°C to the laboratory within 3 h of collection. Groups of 30 to 40 cumulus–oocyte complexes (COCs) were incubated in TCM 199 (Sigma, St. Louis, MO) supplemented with 10% (v/v) fetal calf serum (FCS) (Life Technologies, Gaithersburg, MD), 10 μg/mL FSH (Stimufol, Merial), and 1 μg/mL LH for 22 h at 39°C in a humidified atmosphere of 5% CO_2 in air. At the end of the maturation period, cumulus cells were removed by vortexing and oocytes were enucleated to prepare recipient cytoplasts for cloning. Donor cells were cultured over several passages after thawing to obtain either a growing or a quiescent population of cells on the day of SCNT. The cells were enzymatically dissociated, pelleted, and resuspended in fresh TCM 199. Each isolated cell was inserted under the zona pellucida of the recipient cytoplast and fused by electrofusion.

All the reconstituted embryos were cultured under the same conditions, in microdrops of 50 μL B2 medium (CCD, Paris, France) with 2.5% FCS and seeded with Vero cells. The droplets were overlaid with mineral oil (M8410, Sigma) and incubated for 7 days at 39°C under 5% CO_2. By day 7, expanding or early hatching blastocysts (grades 1 and 2) were transferred into recipients. Recipients were normally cycling Charolais, Normande, or crossbred heifers raised in the same conditions and transported to the experimental farm by the age of 12–14 months after thorough serologic tests to establish the absence of any infectious disease. After estrus detection, heifers that were synchronous $\pm$24 h with embryo age and carrying a palpable corpus luteum were selected for nonsurgical single or double embryo transfer.

Five Holstein cows were used as fibroblast cell donors (genotypes A, C, D, F, G). The mean calving rates for these cell lines were 21.82, 2.44, 8.22, 10, and 26.3% for each genotype, respectively.

Control animals

Holstein heifers and dairy cows from the same experimental farm were inseminated with frozen sperm of Holstein bulls to produce contemporary artificial insemination (AI) female calves.

Monitoring of pregnancy and calving

All recipients were examined for the presence or absence of plasma progesterone at 21 days postovulation. The presence of a viable fetus was detected by day 35 ± 2 days using transrectal ultrasonography (Pie Medical ultrasound with 5.0 Mhz probe). The viability of the fetus as well as the ultrasonographic aspect of the placenta were monitored as previously described (Chavatte-Palmer et al., 2006; Constant et al. 2006). This was performed to detect any pathologic development of pregnancy such as severe hydroallantois. If so, the pregnant recipient was slaughtered, so that only pregnancies with apparently normal fetuses reached term. As a routine procedure for cloned animals, recipients carrying cloned calves were delivered by Cesarean section when natural calving had not occurred by day 282 of pregnancy (all but one recipient) as described before (Chavatte-Palmer et al. 2002). Control animals were born naturally.

Post-natal care

Newborn calves were given pooled colostrum produced on the farm within 2 h after calving. Each cloned calf was paired with a control heifer of approximately the same age (<1 month difference). Calves were isolated in individual stalls up to the age of 2 month and grouped with the rest of the herd afterwards under the same experimental conditions. In this experiment, animals used were born over a period of several years and did not have the same age. Moreover, because of the time frame in which these experiments were performed (1 year), not all animals were subjected to all experiments. The numbers and age ranges of the animals are given for each procedure.

Materials and methods

Blood sampling

Blood was drawn from the tail vein into heparinized vacutainers.

Hematology

Complete blood counts analyses were performed in a commercial medical laboratory using conventional methods. Briefly, total cell counts were performed automatically on a compact automated hematology analyzer design (Cell Dyn 3000, Abott laboratories, Abbott Park, IL), whereas differential leukocyte analyses were performed manually on eosine–nigrosine stained slides.

Preparation of PBMCs

PBMCs were prepared from whole heparinized blood. Blood (60 mL per animal) was centrifuged ($1000 \times g$ for 30 min) at room temperature. The buffy coat interface was recovered and suspended in 5 mL PBS and layered over 20 mL of a 60% Percoll solution (Amersham Bioscience, Piscataway, NJ). Centrifugation was performed at $1000 \times g$ for 30 min at 4°C. The cell pellet was washed three times ($500 \times g$, 10 min, 4°C) in RPMI-1640 (BioWhittaker, Walkersville, MD).

Immunophenotyping of PBMC subsets

The phenotype of PMBCs was studied in nonvaccinated control and SCNT cattle. All immunophenotype assays were done in replicate, usually at less than 1 week intervals. PBMCs (2×10^6) were preincubated in FACS medium (RPMI-1640 containing 4% of horse serum) for 15 min on ice. They were first incubated with 1 mg/mL primary monoclonal antibody (mAb) (Table 1) in FACS medium, washed twice, and further reacted with a 1:200 dilution of fluorescein (FITC)- or phycoerythrin (PE)- or cyanine 5-conjugated goat antimouse (GAM) specific to the immunoglobulin (Ig) isotypes (IgG1, IgG2a, IgM or IgG3, Caltag Laboratories, San Francisco, CA). Irrelevant murine IgG1, IgG2, and IgM (Table 1) were systematically used in order to establish the specificity of subset staining. All the antibodies were monoclonals. The mAbs sold by VMRD have been demonstrated to react with the bovine orthologs of the CD molecules (www.vmrd.com). The anti-CD14 TUK4 mAb has been published to react with the ruminant cluster CD14 (Berthon and Hopkins, 1996). The anti NKp46 AKS1 mAb has been raised against the bovine molecule, and its reactivity with the NK bovine subset has been well characterized (Storset et al., 2004). The DU2-104 mAb

Table 1. Antibodies Used for Immunophenotyping the PBMCs Subsets

Specificity	*Clone*	*Isotype*	*Origin*
CD3 (bovine)	MM1A	IgG1	VMRD[a]
CD4 (bovine)	ILA11	IgG2a	VMRD[a]
CD8 (bovine)	BAQ111A	M	VMRD[a]
CD11b (bovine)	MM12A	IgG1	VMRD[a]
CD14 (human)	TUK4	IgG2a	Santa Cruz[b]
CD25 (bovine)	CACT116A	IgG1	VMRD[a]
CD45R0 (bovine)	ILA116	IgG3	VMRD[a]
γ/δ TcR1 N7 (bovine)	86D	IgG1	VMRD[a]
B cells (unknown molecule, sheep)	DU2-104	IgM	W. Hein[c]
NKp46	AKS1	IgG1	A. Storset[c]
MHC 2 (bovine)	CAT82A	IgG1	VMRD[a]
Isotype control IgG1 Human kappa chain	KP-53	IgG1	Sigma
Isotype control IgG2a Mouse NK cells	NK-1.1	IgG2a	Becton Dickinson
Isotype control IgM Porcine coronavirus	anti-coronavirus	IgM	J. Grosclaude[c]

[a]VMRD, Veterinary Medical Research and Development.
[b]Santa Cruz Biotechnology, Inc.
[c]The mAb were developped by academic laboratories: Wayne Hein (AgResearch, New Zealand), Anne Storset (Norwegian School of Veterinary Science, Norway) and Jeanne Grosclaude (INRA, France).

initially raised against sheep B cells was demonstrated to label all surface immunoglobulin positive cells in cow and is considered as a pan B-cell marker in cow (Mukwedeya et al. 1996).

After two washes in FACS medium, the cells were suspended in Cell-Fix (Becton-Dickinson, Oxnard, CA), and analyzed by flow cytometry using the Cell Quest software (Becton-Dickinson). The analyses were performed on a gate based on size and granularity (FSC/SSC gate) corresponding to mononuclear cells.

Vaccinations

Six pairs (six controls and six clones of the following genotypes: A ($N=1$), C ($N=2$), D ($N=3$), mean age $=11.5\pm3$, range 5–14 months) were vaccinated subcutaneously with a commercial inactivated rotavirus (RV) vaccine (ScourGuard 3, Pfizer, Groton, CT) twice at a 20-day interval (T0 and T20). Seven control and SCNT cattle [clone genotypes: A ($N=2$); F ($N=5$)], (mean age $=10.2\pm4.0$, range 7–17 months), different from the RV group, were vaccinated subcutaneously with 20 mg ovalbumin (OVA, Sigma) in a 1:1 dilution in Alum (Imject Alum, Pierce, Rockville, IL) twice at a 20 days interval (T0 and T20). Sera were collected at T0, T10, and T30. Blood (60 mL) was collected on heparin at T35 for the antigen-specific and mitogen-induced lymphoproliferation assays.

Detection of OVA and RV-specific antibodies

Rotavirus antibody (RV-Ab) and ovalbumin antibody (OVA-Ab) titers in vaccinated cattle were determined by ELISA. Briefly, 96-well plates were coated using cesium chloride-purified RV from the bovine RF strain (200 ng per well (Perrier et al. 2006) or with OVA (1 mg per well) in carbonate buffer. After 1-h saturation in PBS containing 0.05% Tween and 5% fatal calf serum (FCS), serial 1:10 dilutions of serum were added to the wells and incubated for 1 h 30 min at 37°C. After six washes, peroxidase-conjugated antibovine light and heavy chain rabbit IgG (1:2000 dilution, Jackson ImmunoResearch, Avondales PA) was added to wells for 1 h. After washing, the reaction was revealed using TMB (3, 3′, 5, 5′-tetramethylbenzidine) kit reagents (KPL, Gaithersburg, MD) and stopped with HCl (2N). Optical density was measured at 450 nm on a MRX plate reader (Dynex Technologies, Chantilly, PA). The results were expressed as end-point antibody titers calculated with regression analysis by plotting dilutions versus A450 OD (regression curve $y=(b+cx)/(1+ax)$ using Origin software). Endpoint titers were calculated as the inverse of the highest dilution giving twice the absorbance of negative control wells.

Antigen specific and mitogen induced-lymphoproliferation assays

Proliferation assays were carried out in 96-well flat-bottomed plates. Isolated PBMCs (3×10^5 cells per well, triplicates) were seeded in X-vivo 20 serum-free medium including gentamicin alone (BioWhittaker) or with 3 mg/mL purified RV, 500 mg/ml OVA, 10 mg/mL Pokeweed mitogen (PWM, Sigma) or 50 mg/mL phytohemagglutinin (PHA, Sigma). Plates were incubated at 37°C in 5% CO_2 for 96 h, then pulsed overnight with 1 mCi [^{3}H]-thymidine per well. Cells were then collected on filter mats using a cell harvester (Filtermate, Perkin-Elmer, Norwalk, CT) and incorporation of radioactivity into the DNA was measured in a liquid scintillation luminescence counter (Micro beta trilux, Wallac Inc., Gaithersburg, MD) Results were expressed as stimulation indexes (cpm of stimulated cells over cpm of unstimulated cells).

Statistical analysis

Phenotypes and proliferation assays were analyzed using paired Student's *t*-test for each lymphocyte subset (phenotypes) or each condition tested (proliferations). OVA-Ab and RV-Ab responses were analyzed with a PROC-MIXED analysis for repeated measurements using SAS software

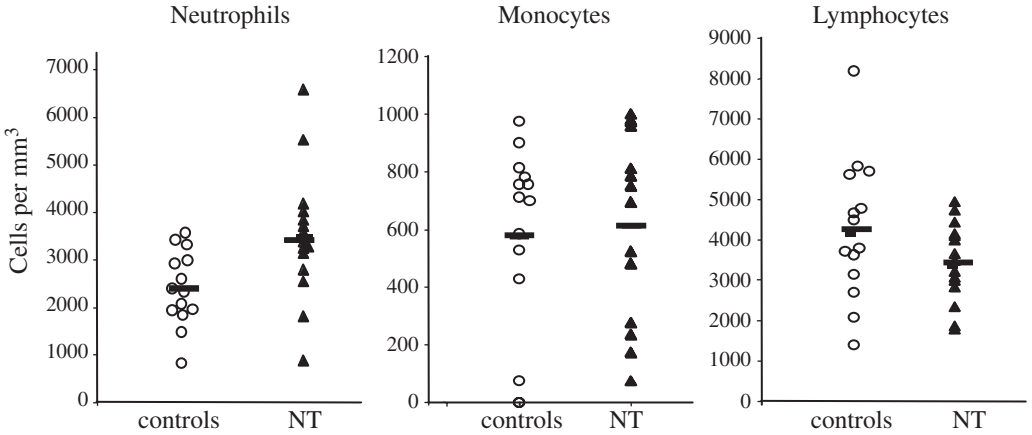


FIG. 1. Blood leukocyte counts in control and SCNT cattle. Neutrophils, monocytes and lymphocytes numbers (cell counts per mm^3) were evaluated by conventional hematological methods in controls ($n = 15$) and SCNT ($n = 15$) cattle. All values were within the normal range and no statistically significant differences could be found between control and SCNT values ($p = 0.06$, 0.74 and 0.13 for neutrophils, monocytes, and lymphocytes, respectively).

(Littel et al., 1998). The response at a fixed time (30 days after the second booster) was also analyzed using a Student's *t*-test. The effect of clone genotype was not analyzed due to the small number of animals.

Ethical approval

The experiment was performed in accordance with the International Guiding Principles for Biomedical Research involving Animals as promulgated by the Society for the Study of Reproduction, and with the European Convention on Animal experimentation. All researchers involved with direct work with the animals possess an animal experimentation license (level 1 for principal investigator or level 2 for technicians) delivered by the French veterinary services. Research work on cloned animals was approved by the INRA ethical committee (COMEPRA) in December 1999.

Results

SCNT and control cattle, studied past the neonatal period, present similar survival rates

The experiments were performed in 2004–2005 on apparently healthy animals. During the 4 following years, three control and four cloned (genotypes A, D, and G) animals were culled. In the control group, the cows were culled due to unsufficient milk production ($N = 1$), peritonitis ($N = 1$) and for lameness ($N = 1$), whereas in the clone group, the animal from the G genotype and two cows from the A genotype were culled for lameness, whereas one animal from the D genotype was put down after it fell and broke its leg. Furthermore, one clone of the D genotype died in 2004 with undiagnosed pathology.

Blood granulocytes and PBMCs subsets

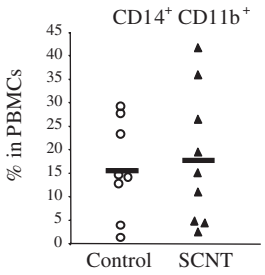
Fifteen SCNT cattle from five distinct genotypes and 15 age-matched controls were used to probe for any abnormalities in the representation of the leukocyte subsets that could be related to cloning. A classical hematological analysis (Fig. 1) showed that the neutrophils counts in blood were similar between control and SCNT cattle (range = 814–3570 cells per mm^3 and 864–6560 cells per mm^3, respectively). Variations according to age were also found. They were within the normal range in both groups as previously reported in young (Chavatte-Palmer et al., 2002) and adult clones (Heyman et al., 2007b). Very low numbers of eosinophils (0–700 per mm^3) and basophils (0–100 per mm^3) were found in the two groups (data not shown). In addition, monocytes were found in similar quantities in control and SCNT cattle (range = 0–900 cells per mm^3 and range = 73–1000 cells per mm^3, respectively; Fig. 1) as were lymphocytes (ranges = 1400–5700 cells per mm^3 and 1700–4900 cells per mm^3, respectively; Fig. 1). Within PBMCs, several cell subsets were analyzed that are involved in innate immunity (monocytes, NK, and g/d T cells) and acquired immunity (a/b T cell and B subsets). Within a replicate, cell proportions were always within the same range and the mean of the two experiments was used for the statistical analyses.

Monocytes ($CD14^+$ $CD11b^+$ cells) showed a similar range of representation in control and SCNT PBMCs (range = 1.29–29.25% and 2.4–35.8%, respectively; Fig. 2A), supporting and confirming the hematological results (Fig. 1).

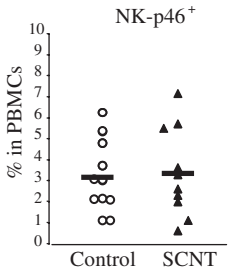
NK cells were analyzed using the expression of p46-NK (Storset et al., 2004) (Fig. 2B). The % p46-NK^+ cells were similar in control cattle PBMCs (range = 1.17–6.23%) and in SCNT PBMCs (range = 0.65–7.1%).

Regarding the T lymphocytes (Fig. 2C), their proportions in blood were similar in control cattle (range = 15.5–52%) and in SCNT cattle (range = 16.4–51%). The TcR1-N7+ g/d T cells, corresponding to $WC1^+$ γ/δ T-cell subset (Davis et al., 1996), were similarly represented in control and SCNT cattle (ranges = 1.17–10.7% and 0.58–17.5%, respectively). Regarding α/β T cells, the $CD4^+$ T cell proportions were similar in control (range = 2.7–39.8%) and in SCNT cattle (range = 6–30.9%) as were the $CD8^+$ T cells (range = 1.2–21.8% in controls and range = 3.2–16.3% in SCNT). In order to deepen the analysis, we evaluated the proportion of specialized T-cell subsets. We found that $CD4^+$ $CD25^+$ T cells, that display regulatory functions in cattle, were similarly represented within $CD4^+$ T cells in control (range = 2.3–18%) and SCNT cattle (range = 2.8–21.4%). Also, the activated MHC-II^+ $CD4^+$ T cells showed similar proportions within the $CD4^+$ T cells of control (range = 3.1–31%) compared to cloned cattle

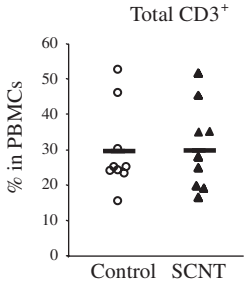
A Monocytes

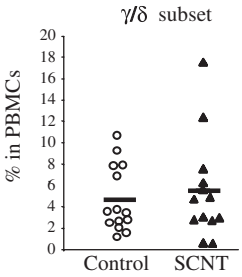


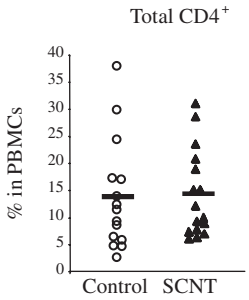
B NK

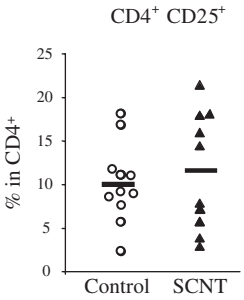


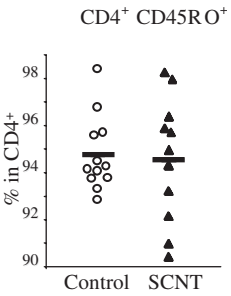
C T lymphocytes

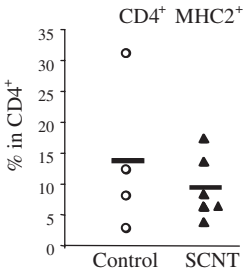


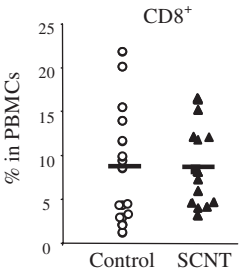


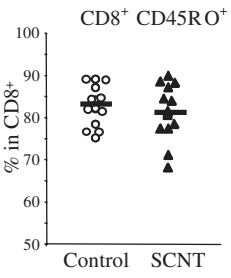




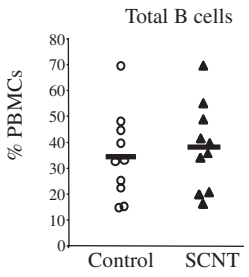


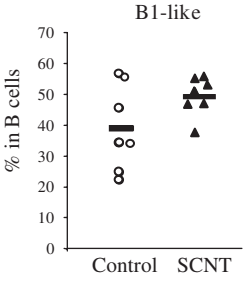






D B lymphocytes





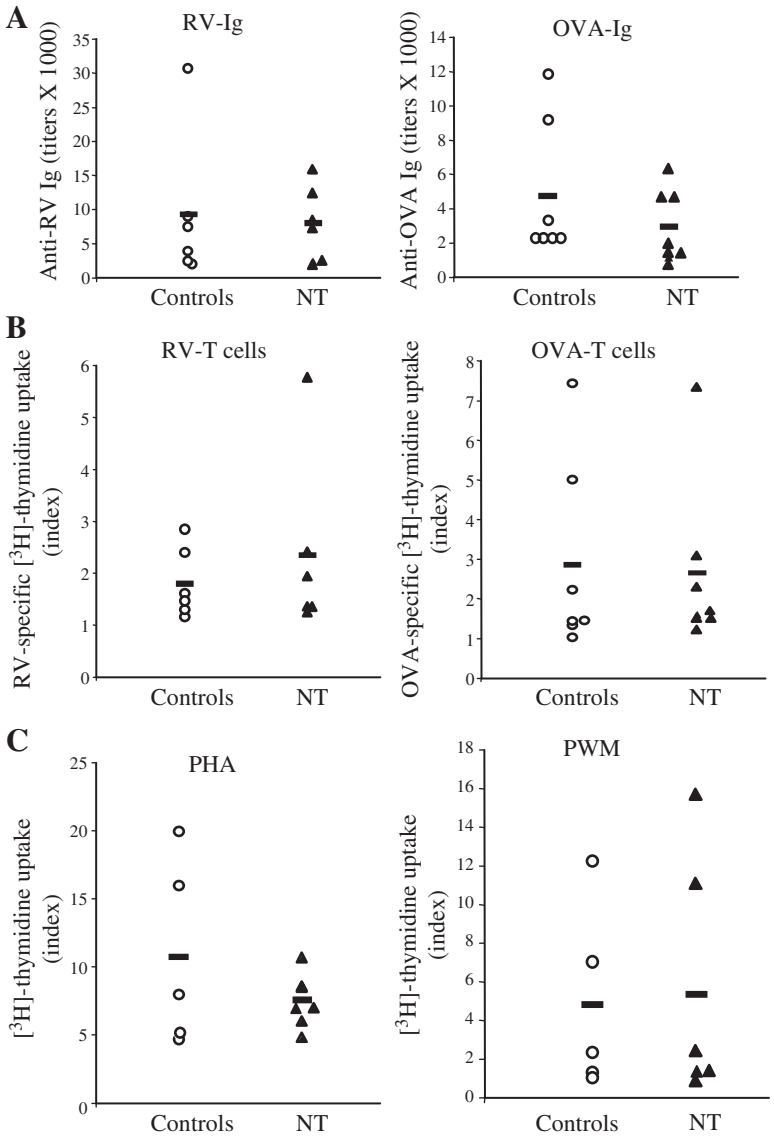


FIG. 3. Analysis of immune responses in control and SCNT cattle. Six control and SCNT cattle were vaccinated twice (D0 and D20) with inactivated bovine RV (RV group). Seven control and SCNT cattle, distinct from the RV group, were vaccinated twice (D0 and D20) with OVA (OVA group). (**A**) RV- and OVA-specific serum Ig were measured by ELISA on D30. No statistical difference was found between the control (empty circle) and SCNT (filled triangle) cattle ($p = 0.80$ and 0.78 for RV and OVA, respectively). (**B**) PBMCs were collected on day 35 and restimulated *in vitro* with 3 mg/mL purified RV (RV group) or 500 mg/mL OVA (OVA group). [^{3}H]-Thymidine incorporation was done on the last 18 h of a 5-day culture. Stimulation indexes are shown. No statistical difference was found between the control and SCNT cattle ($p = 0.23$ and $p = 0.48$ for RV and OVA, respectively). (**C**) PBMCs were collected from five control and six SCNT cattle and they were stimulated *in vitro* with PHA (50 mg/mL) and PWM (10 mg/mL). [^{3}H]-Thymidine incorporation was done on the last 18 h of a 48-h culture. Stimulation indexes are shown. No statistical difference was found between the control and SCNT cattle ($p = 0.16$ and 0.43 for PHA and PWM, respectively).

FIG. 2. Analysis of PBMCs subsets in control and SCNT cattle. Subset representations (%) were established in control (empty circle) and SCNT (filled triangle) PBMCs. (**A**) Monocytes were defined as CD14$^+$ CD11b$^+$ cells ($n = 8$ in control and $n = 9$ in SCNT cattle). (**B**) NK cells were evaluated as CD3$^-$ CD2$^+$ ($n = 9$ in control and $n = 5$ in SCNT cattle) or NK-p46$^+$ cells ($n = 11$ in control and $n = 10$ in SCNT cattle). (**C**) T lymphocytes were identified as CD3$^+$ cells ($n = 9$ in control and SCNT cattle) and the analyzed T cell subsets were: a subset of γ/δ T cells (TcR1 N7$^+$, $n = 14$ in control and 13 in SCNT cattle), the CD4$^+$ T cells ($n = 15$ in controls and $n = 16$ in SCNT cattle) including regulatory CD4$^+$ CD25$^+$ T cells ($n = 11$ in control and $n = 10$ in SCNT cattle), memory CD4$^+$ CD45RO$^+$ T cells ($n = 12$ in control and SCNT cattle) and activated CD4$^+$ MHC-II$^+$ T cells ($n = 4$ in control and $n = 6$ in SCNT cattle), the CD8$^+$ T cells (15 in control and 14 in SCNT cattle) including memory CD8$^+$ CD45RO$^+$ T ($n = 12$ in control and SCNT cattle), cells and (**D**) B lymphocytes ($n = 10$ in control and SCNT cattle) including the CD11b$^+$ B1-like subset ($n = 7$ in control and SCNT cattle). No statistically significant differences could be found between control and SCNT groups for any analyzed subset (p-value ranges: from 0.08 to 0.99).

(range = 4.1–17%). Finally, no differences were found between the representation of memory T cells both in the $CD8^+$ and $CD4^+$ T populations ($CD4^+$ $CD45RO^+$ in controls: range = 92–98% and $CD4^+$ $CD45RO^+$ in clones: range = 90–98%; $CD8^+$ $CD45RO^+$ in controls: range = 75–89% and $CD8^+$ $CD45RO^+$ in clones: 71–89%).

B lymphocytes (Fig. 2D) were as represented in control as in SCNT cattle PBMCs (range = 15–69% and 16–69%, respectively). Furthermore, the B1-like cell representation ($CD11b^+$ B) showed any significant differences between control and SCNT cattle (range = 22–58% and 36–54%, respectively).

In conclusion, no alterations in any leukocyte subset representation could be detected in cloned cattle.

Humoral and cellular responses to OVA and RV and lymphocyte proliferative responses to mitogens

In order to test whether SCNT cattle showed altered immune responses, SCNT cattle, and age-matched controls (six per group) were vaccinated twice with a commercial inactivated vaccine against RV. Other SCNT and age-matched controls (seven per group) were also vaccinated twice with an inert classical antigen, that is, OVA in alum. Ten days after the boost injection, the total anti-RV Ig in serum reached similar titers in control and SCNT cattle (range = $2–30\times10^3$ and $2–15\times10^3$, respectively; Fig. 3A). The same observation was obtained with the anti-OVA humoral response (titers in controls: range = $2–11\times10^3$ and in SCNT cattle: range = $0.6–6.2\times10^3$; Fig. 3A). Regarding the T-cell stimulation assay, a low RV-specific T-cell response was measured by $\{^3H\}$-thymidine uptake both in control and SCNT cattle showing no significant differences between the two groups (index range in controls = 1.25–2.6 and in SCNT = 1.35–5.3; Fig. 3B). A low specific OVA T-cell response was also detected in both groups (index range in controls = 1.3–7.4 and in SCNT = 1.2–7.3; Fig. 3B). Finally, both SCNT and control groups from the RV experiment presented similar lymphocyte response to PWM, a T and B lymphocyte mitogen (range in controls = 1–12 and range in SCNT cattle = 1–15.6; Fig. 3C) and to PHA, a T lymphocyte mitogen (range in controls = 4.6–20 and range in SCNT cattle = 4.8–10.6; Fig. 3C).

In conclusion, SCNT cattle developed normal antibody and T-cell responses to two specific antigens. In addition, the PBMCs from SCNT cattle were stimulated by polyclonal mitogens as efficiently as the PBMCs from control cattle.

Discussion

We could not detect any alteration in the immune parameters measured in a large number of cloned cattle when compared to age-matched controls. Indeed, a thorough comparison of the blood leukocyte subset representation in cloned and control cattle did not reveal differences that would indicate alterations in the leukocyte subset development as a result of cloning. Furthermore, functional immunity was not modified in cloned cattle, as they were as capable as controls to develop B- and T-cell-specific responses against the model antigen OVA and against RV vaccine. Thus, this analysis supports the fact that cloned cattle have a normal immunity.

No differences could be detected in the proportions of the different T and B lymphocyte subsets between control and SCNT cattle. However, the markers that we used for "subsetting" do not always perfectly delineate a functional subset. For instance, the $CD4^+$ $CD25^+$ T cells in cattle were functionally characterized as Treg cells in two studies (Seo et al., 2007), although it is well known that these markers are not sufficient to detect all Treg in the mouse (Lages et al., 2008), and that CD25 is upregulated during T-cell activation. Unfortunately, several mAb directed to human FoxP3, a transcription factor crucial for Treg differentiation and function (Khattri et al., 2003), did not give a reliable staining of bovine T cells in our hands. In any event, the $CD4^+$ $CD25^+$ T cells represented around 10% of the CD4 T cells in control and SCNT cattle, similar to what has been found in the mouse (Lages et al., 2008) and in humans (Jonuleit et al., 2001). Also, not all g/d T cells were identified by the anti TcR1-N7 mAb that mainly labels the WC1 g/d subset that corresponds to an activated, proliferative and inflammatory γ/δ subset (Meissner et al., 2003). Interestingly, this subset was strongly reduced in the PBMCs of cloned cows in early lactation, but was back to normal values during the rest of the lactation period (Tanaka et al., 2006), in accordance with our results. Finally $CD4^-$ $CD8^-$ T subsets with invariant T-cell receptor conserved in mice and humans [NK-T cells and MR1-restricted mucosal associated invariant cells (MAIT)] were not analyzed in our study due to lack of knowledge and available markers in cattle. NKT cells may not exist in cattle (Van Rhijn et al., 2006), but MAIT cells have been detected (Treiner and Lantz, 2006).

The preservation of immune parameters observed in our study is in accordance with the global good health of our cloned cattle. However, a higher incidence of diseases was observed in SCNT animals in a New Zealand study (Wells et al., 2004). This discordance may be due to the fact that we are reporting survival rates on a limited number of animals compared to Wells at al., and that housing conditions at INRA (indoor housing, few lactations) are less demanding for the animals compared to the outdoor conditions with repeated lactations in the New Zealand herd. Moreover, as discussed above, there might be genotype-related effects, or differences due to the nuclear transfer method.

In view of the recent debate on the health of animal clones, the data presented here confirm that the immune system is not depressed in cloned cattle. The fragile health reported in animal clones (Wells, 2005) may not be directly related to a deficit in the immune system but rather to an overall weakness that may only be revealed when the animals are under heavy stress. Indeed, preliminary work indicates that there are altered hypothalamic–pituitary–adrenal axis responses to a glucagon test and reduced adrenal sensitivity in 6-month-old cloned Jersey bull calves (Green et al., 2008). Moreover, anatomical differences have been observed in cloned bulls with lighter lungs and brains and heavier thymus, thyroids, testis, and femurs relatively to contemporary controls (Green et al., 2007). It is important to consider that these differences may be directly due to the cloning process with abnormal epigenetic apposition at the time of nuclear reprogramming, or they may result from the epigenetic programming of the fetus due to the observed abnormal intrauterine development and abnormal placentation (Constant et al., 2006; Hill et al., 2000), as shown after intrauterine growth retardation in humans and animal models (Gluckman and Hanson, 2004; Gluckman et al., 2007). In any case, the immune system appears to be sufficiently robust so that the animals that survive to adulthood seem to

possess normal immune responses, as attested by our observation using nonreplicative vaccines. It is also possible that the interindividual heterogeneity masks existing alterations. In addition, an infectious challenge with parasites, viruses, and bacteria in SCNT cattle could be done to further understand their capacity to resist to infectious diseases and to mount protective immunity. Indeed, under an aggression by pathogens, an SCNT animal might reveal an otherwise undetectable immune defect. In any case, the data presented here also reflect only the immune status of the animals at a set moment in time and cannot preclude of possible variations due to environmental factors at later ages.

As mentioned earlier, SCNT is compatible with the birth of live offspring in a wide range of mammalian species, but the overall efficiency of this technology is low. High rates of embryonic and fetal mortality, and an increased incidence of congenital defects, have been linked with perturbations in developmentally important epigenetic marks such as DNA methylation and histone modifications (Dean et al., 2003; Santos et al., 2003). Calves that survive appear to have normal epigenetic marking of some key imprinted genes marking (Yang et al., 2005), but recent data from our lab shows that the functional reprogramming of a given donor genotype is compatible with a highly flexible methylation status of its DNA; thus, genomic copies of adult animals have to be considered as epigenome variants (Montera et al., 2009, personal communication) presenting phenotypic variability that may apply to immune parameters. This could explain that in this study as in other studies on the phenotype of clones, the variability between clones of the same genotype appears to be the same if not more than between control animals.

Agronomical applications of animal clones mainly include the production of genetically highly valuable animals to be used for breeding (Westhusin et al., 2001). The production of transgenic animals through cloning is another avenue for the cloning technology (Yang et al., 2007). The preserved immune parameters that we observed in adult bovine clones is very important for the development of cloning in the industry and to build up confidence of the general public in new biotechnologies.

The results obtained here, comforted by the fact that most animals used 4 years ago in this study are still alive, regardless of whether they were produced by conventional reproduction or by cloning, are very important as scientific references to be used by national agencies to possibly justify the safety of the cloning procedure, especially in the European context where most Europeans object the use of cloned animals (Flash, 2008).

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Author Disclosure Statement

The authors declare that no conflicting financial interests exists.

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