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Antigen-specific human polyclonal antibodies from hyperimmunized cattle

Yoshimi Kuroiwa[1,2], Poothappillai Kasinathan[1], Thillainayagen Sathiyaseelan[1], Jin-an Jiao[1], Hiroaki Matsushita[1], Janaki Sathiyaseelan[1], Hua Wu[1], Jenny Mellquist[1], Melissa Hammitt[1], Julie Koster[1], Satoru Kamoda[2], Katsumi Tachibana[2], Isao Ishida[2] & James M Robl[1]

Antigen-specific human polyclonal antibodies (hpAbs), produced by hyperimmunization, could be useful for treating many human diseases. However, yields from available transgenic mice and transchromosomic (Tc) cattle carrying human immunoglobulin loci are too low for therapeutic applications. We report a Tc bovine system that produces large yields of hpAbs. Tc cattle were generated by transferring a human artificial chromosome vector carrying the entire unrearranged, human immunoglobulin heavy (h*IGH*) and κ-light (h*IGK*) chain loci to bovine fibroblasts in which two endogenous bovine *IgH* chain loci were inactivated. Plasma from the oldest animal contained >2 g/l of hIgG, paired with either human κ-light chain (up to ~650 μg/ml, fully human) or with bovine κ- or λ-light chain (chimeric), with a normal hIgG subclass distribution. Hyperimmunization with anthrax protective antigen triggered a hIgG-mediated humoral immune response comprising a high proportion of antigen-specific hIgG. Purified, fully human and chimeric hIgGs were highly active in an *in vitro* toxin neutralization assay and protective in an *in vivo* mouse challenge assay.

hpAbs, produced from donated human plasma, have been used therapeutically for many years[1,2]. In an effort to improve effectiveness for specific disease applications, some products have been made in immunized humans[3], despite substantial challenges and restrictions. These include limitations on the types of vaccines used, number of immunizations permitted, types of adjuvant, amount of plasma that can be collected and dependence on voluntary donations. Alternatively, human plasma donors have been screened to select those with naturally high reactivity to specific antigens. Because hpAbs could be useful for treating many life-threatening human diseases, such as bacterial and viral infections, cancer and various autoimmune syndromes, an alternative hpAb production system is greatly needed[4,5].

Transgenic mice carrying the human immunoglobulin loci produce antigen-specific hpAbs in response to hyperimmunization[4], demonstrating that the mouse immune system can support human immunoglobulin gene rearrangement, affinity maturation and human antibody production following hyperimmunzation. Although human antibody–producing mice are ideal for generating human monoclonal antibodies, their small body size makes them unsuitable for producing practical amounts of therapeutic hpAbs. Large farm animals, such as cattle, could be a desirable source for therapeutic hpAbs because their size would enable them to produce a large quantity of antibodies after hyperimmunization with desired antigens.

Previously, we reported the generation of transchromosomic (Tc) cattle carrying a human artificial chromosome (HAC) vector comprising the entire, germline-configured, h*IGH* and h*IGL* chain loci[6]. Although human immunoglobulin gene rearrangement appeared normal in Tc cattle, the level of hIgG produced in their plasma was very low (~10 μg/ml). We suspect that dominant expression of endogenous bovine IgG (bIgG) suppressed expression of hIgG. In mice transgenic for human immunoglobulin, disruption of endogenous murine immunoglobulin genes by gene targeting resulted in a significant increase in production of hIgG[4]. Therefore, inactivation of the endogenous bovine immunoglobulin gene(s) could enhance production of hpAbs in Tc cattle.

In comparison with those of mouse and human, little is known about immunoglobulin gene function and organization in cattle. Among IgH chain classes, the IgM heavy chain of mouse and human is encoded by a single gene, *IGHM*, which is the first to be expressed during early B cell development and is essential for B-cell development[7–9]. In contrast, large farm animals, such as sheep, goat and cattle, appear to possess two IgM loci[10]: the classical *IGHM* as well as an IgM-like (*IGHML1*) locus. In cattle, two distinct IgM sequences have been registered: U63637 (or AY149283) encodes *IGHML1* (located on chromosome 11; refs. 11–14), whereas AY230207 (or AY158087) encodes *IGHM* (mapped to chromosome 21; refs. 14,15). Although it is unknown whether the additional *IGHML1* locus is functional, if it supports B-cell development and IgG production in the absence of *IGHM*, then two heavy-chain gene knockouts (four targeting events) would be required to inactivate bovine immunoglobulin production.

Another potentially challenging problem associated with the use of a Tc bovine hpAb-production system is whether or not the human

[1]Hematech, Inc, 4401 S. Technology Dr., Sioux Falls, South Dakota 57106, USA. [2]Kyowa Hakko Kirin Co., Ltd., 1-6-1 Ohtemachi, Chiyoda-ku, Tokyo 100-8185, Japan. Correspondence should be addressed to J.M.R. (jrobl@hematech.com) or Y.K. (ykuroiwa@hematech.com).

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immunoglobulin genes could support bovine B-cell development and humoral immunity in the absence of functional bovine immunoglobulin gene expression. Because the immune system in large farm animals is distinctly different from that of the mouse and human[16–22], successful production of hpAbs in the mouse is not necessarily indicative of success in cattle.

In this study, we first addressed the question of *IGHML1* function by generating and evaluating a series of IgM knockout cattle. We found that, surprisingly, each of the two IgM loci is fully functional and inactivation of both IgM loci is required for complete B-cell deficiency in cattle. Second, we investigated the function of a HAC vector (κHAC) comprising both h*IGH* and h*IGK* loci, in IgM double-knockout ($IGHM^{-/-}IGHML1^{-/-}$) cattle. We report here a detailed characterization of our first, κHAC/$IGHM^{-/-}IGHML1^{-/-}$ calf. Production of this calf (468) required five sequential genetic modifications and seven consecutive cloning events. Calf 468 continuously produced >2 g/l of hIgG in plasma, 10–20% of which (up to 649.1 μg/ml) was fully human hIgG (hIgG/hκ-chain). After hyperimmunization with anthrax protective antigen (PA), both fully human hIgG/hκ-chain and chimeric hIgG antibodies were found to be highly effective in an *in vitro* toxin-neutralization assay (TNA) and in an *in vivo* mouse protection assay. These results demonstrate the feasibility of using a bovine system to produce a large volume of highly active hpAbs for human therapy.

RESULTS

Generation and analysis of $IGHM^{-/-}$, $IGHML1^{-/-}$ and $IGHM^{-/-}IGHML1^{-/-}$ knockout cattle

We previously generated IgM knockout cattle by using a sequential gene targeting system, based on the U63637 sequence, which was the only one registered at that time[23]. By constructing and screening a genomic library made from the IgM knockout bovine fibroblast cell line, we found that our previous IgM knockout was indeed $IGHML1^{-/-}$; both alleles of *IGHML1*, designated as alleles *U* and *u*, were disrupted by the knockout cassettes, whereas the *IGHM* alleles, designated as *AY* and *ay*, were still intact (**Supplementary Fig. 1a** online).

To elucidate the involvement of both IgM loci, *IGHM* and *IGHML1*, in B-cell development in cattle, we generated $IGHML1^{-/-}$, $IGHM^{-/-}$ and $IGHM^{-/-}IGHML1^{-/-}$ knockout animals. To specifically knock out both alleles of the *IGHM* gene, we constructed the allele-specific knockout vectors pbCμayKOhyg and pbCμAYKObsr (from the alleles *ay* and *AY*, respectively), which were identified from the genomic library used previously[23] (**Supplementary Fig. 1b**). The wild-type bovine fibroblast line 6939 was transfected with pbCμayKOhyg to target allele *ay*, and $IGHM^{-/+}$ colonies were identified by PCR. Seventeen $IGHM^{-/+}$ colonies were identified from 210 (8.1%) hygromycin B-resistant colonies. To rejuvenate cells, we produced cloned embryos, collected four 40-d cloned fetuses and established fibroblast cell lines. All four cell lines were confirmed to be $IGHM^{-/+}$ by genomic PCR (**Supplementary Fig. 1c**). Evaluation of a polymorphic sequence within the PCR products demonstrated that the vector was exclusively integrated into allele *ay* of the *IGHM* gene in all four fetuses. One $IGHM^{-/+}$ cell line was then subjected to a second round of gene targeting to disrupt the second allele, *AY*, of *IGHM*, using a second knockout vector (pbCμAYKObsr). Fourteen $IGHM^{-/-}$ colonies were identified from 146 (9.6%) blasticidine-resistant colonies. After embryonic cloning of colonies, six rejuvenated cell lines were produced from fetuses recovered at 40 d. All proved to be $IGHM^{-/-}$ by genomic PCR (**Supplementary Fig. 1d**). Sequence analysis of the PCR products (AYKObsrF2 × AYKObsrR2) demonstrated that the second knockout vector was exclusively integrated into allele *AY* of the *IGHM* gene in all six fetuses. To generate the double-knockout $IGHM^{-/-}IGHML1^{-/-}$ cell lines, we further transfected the $IGHML1^{-/-}$ cell line established previously[23] with the knockout vectors (pbCμayKOhyg and pbCμAYKObsr) to sequentially disrupt the two alleles, *ay* and *AY*, of the *IGHM* gene. After two additional rounds of gene targeting (29 $IGHM^{-/+}IGHML1^{-/-}$ colonies were identified from 453 (6.4%) hygromycin B-resistant colonies; 26 $IGHM^{-/-}IGHML1^{-/-}$ colonies were identified from 215 (12.1%) blasticidine-resistant colonies), four fetuses were collected at 40 d and shown to be $IGHM^{-/-}IGHML1^{-/-}$ by genomic PCR (**Supplementary Fig. 1e**). Targeting frequencies at *IGHM* were substantially higher than those at the *IGHML1* locus (0.17–0.45%)[23], presumably due to use of allele-specific targeting vectors.

To verify specific disruption of each of the genes, we evaluated expression by RT-PCR analysis (primers; BL17 × mBCμR2) on spleen cells from $IGHM^{-/-}$, $IGHML1^{-/-}$, $IGHM^{-/-}IGHML1^{-/-}$ and wild-type control fetuses after 180 d of gestation (**Supplementary Fig. 1f**). All fetuses originated from the same primary bovine fibroblast line 6939, as described above. After sequence analysis of the amplified transcripts, we confirmed specific disruption of *IGHM* or *IGHML1* gene expression and expression of *IGHML1* or *IGHM*, in the $IGHM^{-/-}$ or $IGHML1^{-/-}$ fetuses, respectively. Gene expression was not detected from either of the two IgM genes in $IGHM^{-/-}IGHML1^{-/-}$ fetuses (**Supplementary Fig. 1g**). Although both *IGHM* and *IGHML1* transcripts were detected in wild-type fetuses, the level of expression of *IGHML1* appeared to be much lower than that of *IGHM*, indicating that *IGHML1* is a minor IgM class in the presence of *IGHM* in wild-type cattle.

$IGHM^{-/-}$, $IGHML1^{-/-}$ and $IGHM^{-/-}IGHML1^{-/-}$ cell lines were used to generate calves (**Table 1**) for comparison of B-cell development, immunoglobulin protein secretion and antigen-specific humoral immune response. Flow-cytometry analysis of peripheral blood mononuclear cells showed clear B-cell populations ($CD21^+$,

Table 1 Production of cloned calves from genetically modified fibroblast cell lines

Cell line ID	Genotype	Recipients	Pregnant at (%)[a]				Calves survived more than 2 months (%)[a]
			40 d	90 d	150 d	270 d	
F056-2	$IGHM^{-/-}$	62	34 (55)	21 (40)	20 (38)	19 (37)	15 (29)
1638	$IGHM^{-/-}IGHML1^{-/-}$	49	23 (47)	9 (18)	7 (14)	2 (4)	2 (4)
261R	κHAC/$IGHM^{-/-}$	454	261 (57)	113 (25)	95 (21)	68 (15)	71 (16)
A254-2	κHAC/$IGHM^{-/-}IGHML1^{-/-}$	37	23 (62)	11 (30)	7 (19)	2 (5)	0 (0)
443	κHAC/$IGHM^{-/-}IGHML1^{-/-}$	213	84 (39)	6 (3)	5 (2)	2 (1)	1 (0.5)
Total		815	425 (52)	160 (20)	134 (17)	93 (12)	90 (11)

[a]Percentages were calculated by dividing the number of fetuses or calves by that of recipients implanted.

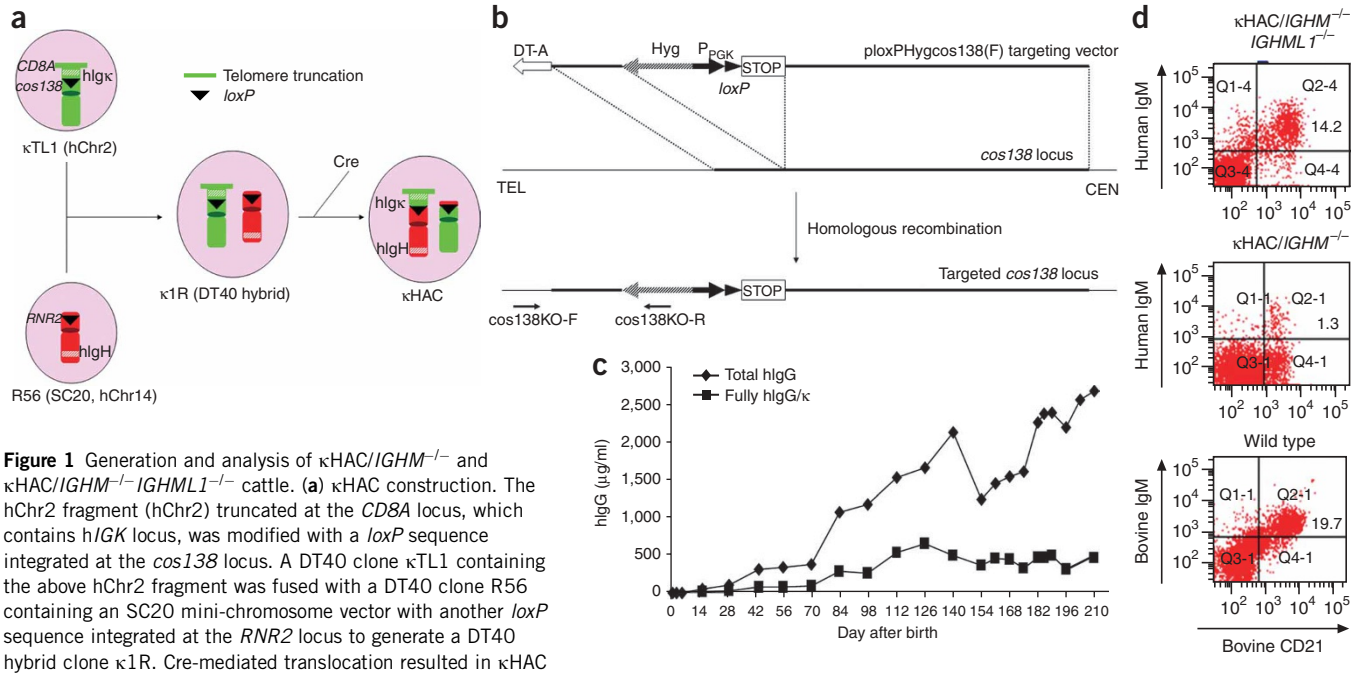

Figure 1 Generation and analysis of κHAC/*IGHM*$^{-/-}$ and κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ cattle. (**a**) κHAC construction. The hChr2 fragment (hChr2) truncated at the *CD8A* locus, which contains h*IGK* locus, was modified with a *loxP* sequence integrated at the *cos138* locus. A DT40 clone κTL1 containing the above hChr2 fragment was fused with a DT40 clone R56 containing an SC20 mini-chromosome vector with another *loxP* sequence integrated at the *RNR2* locus to generate a DT40 hybrid clone κ1R. Cre-mediated translocation resulted in κHAC comprising both entire *hIGH* and h*IGK* loci. (**b**) Targeting vector ploxPHygcos138(F). The vector comprises a 5′ homologous arm, a 3′ homologous arm, STOP cassette containing transcriptional and translational stop sequence, a *loxP* sequence, PGK promoter, DT-A (diphtheria toxin A gene) and *hyg* gene. A primer pair, cos138KO-F x cos138KO-R, was used to identify the event of homologous recombination. (**c**) Total hIgG and fully human hIgG/hκ-chain (hIgG/κ) levels in calf 468 serum for 7 months after birth. (**d**) B-cell population in Ileal Peyer's patch of κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$, κHAC/*IGHM*$^{-/-}$ and control bovines, stained with anti-CD21 and hIgM (or bIgM) antibodies.

IgM$^+$B220$^+$) in both *IGHM*$^{-/-}$ and *IGHML1*$^{-/-}$ calves, whereas no B cells were detected in *IGHM*$^{-/-}$*IGHML1*$^{-/-}$ calves (**Supplementary Fig. 1h**). B220$^+$IgM$^-$ cells were detected in *IGHM*$^{-/-}$*IGHML1*$^{-/-}$ calves and could be pro-B cells (the stage before IgM cell surface expression), because an IgM knockout can not ablate pro-B-cell generation. We have also performed RT-PCR analysis for $V_HD_HJ_H$-rearranged bovine IgD and IgG transcripts and were not able to detect the transcripts in *IGHM*$^{-/-}$*IGHML1*$^{-/-}$ bovines, suggesting that the B220$^+$IgM$^-$ cells are neither IgD$^+$ nor IgG$^+$ B cells. Furthermore, the cells were not CD21$^+$, which should be the case for either IgD$^+$ or IgG$^+$ B cells. Within the first day after birth, before colostrum administration, we detected secreted IgM protein in sera of the *IGHM*$^{-/-}$ (4–11 μg/ml) and the *IGHML1*$^{-/-}$ (4–7 μg/ml) calves, at levels comparable to controls (8–21 μg/ml). No secreted IgM protein was detected in the *IGHM*$^{-/-}$*IGHML1*$^{-/-}$ calves (the detection limit of this enzyme-linked immunosorbent assay (ELISA) is 0.4 μg/ml). IgG protein was detected in the sera of the *IGHM*$^{-/-}$ (8–11 μg/ml), *IGHML1*$^{-/-}$ (6–11 μg/ml) and, surprisingly, in *IGHM*$^{-/-}$*IGHML1*$^{-/-}$ (4–11 μg/ml) calves. The IgG protein detected in sera of *IGHM*$^{-/-}$*IGHML1*$^{-/-}$ calves is likely to have come from the mother, possibly through the placenta, because *IGHG* transcripts were not detected in peripheral blood mononuclear cells (**Supplementary Fig. 1i**).

When calves were 3–4 months of age, we detected high levels of IgG protein in the *IGHM*$^{-/-}$ and *IGHML1*$^{-/-}$ calves (30–42 mg/ml and 34–41 mg/ml, respectively). Furthermore, both types of calves responded to immunization with titers comparable to wild-type controls (**Supplementary Fig. 1j,k**). These data demonstrate that, in contrast to the mouse and human, cattle possess two fully functional IgM loci, *IGHM* and *IGHML1*, each capable of supporting B-cell development and antigen-specific humoral immune response. For a complete inactivation of immunoglobulin gene function in cattle, both loci need to be disrupted.

Generation and analysis of κHAC/*IGHM*$^{-/-}$ and κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ cattle

Previously[6], we introduced a HAC, carrying both h*IGH* and h*IGK* chain loci (ΔΔHAC), into cattle to produce hIgG. To improve the level of expression of hIgG, we considered constructing a different HAC for this study. As rearrangement and expression of the *IGK* locus precedes that of the λ-light chain locus (*IGL*)[24] in human and mouse, the h*IGK* locus might compete with bovine immunoglobulin light chain loci (b*Igl*) better than the h*IGL* locus because the immunoglobulin λ-light chain is the predominant light chain expressed in cattle[21]. Furthermore, human κ-chain normally represents more than half of the total human immunoglobulin light chain (κ/λ ratio = 60/40)[25] expressed in human. Based on this rationale, we attempted to construct a HAC vector comprising the entire loci for both h*IGH* and h*IGK* chain genes (κHAC) using a chromosome-cloning system[26] (**Fig. 1a,b**). The κHAC was introduced into either *IGHM*$^{-/-}$ or *IGHM*$^{-/-}$*IGHML1*$^{-/-}$ bovine fibroblasts by microcell-mediated chromosome transfer and calves were generated by embryonic cloning (**Table 1**).

The κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ cell line (A254-2) generated calves at lower efficiency than the κHAC/*IGHM*$^{-/-}$ cell line (**Table 1**), possibly because of the additional two rounds of embryonic cloning (total of six) required to knock out both IgM loci. Of the two male calves produced, calf 445 died shortly after birth, whereas calf 443 survived to 40 d and produced 541 μg/ml of total hIgG (fully human hIgG/hκ-chain + chimeric) in the serum. This level was substantially higher than that in our previous ΔΔHAC calves (~10 μg/ml). We established a fibroblast cell line from calf 443 and conducted an additional (seventh) round of embryonic cloning, which gave rise to one healthy calf, 468. The scheme for generation of calf 468 is summarized in **Supplementary Figure 2** online.

From birth, calf 468 showed a substantial increase in hIgG; reaching > 1 g/l in serum at 84 d of age (**Fig. 1c**). Human IgM was also detected

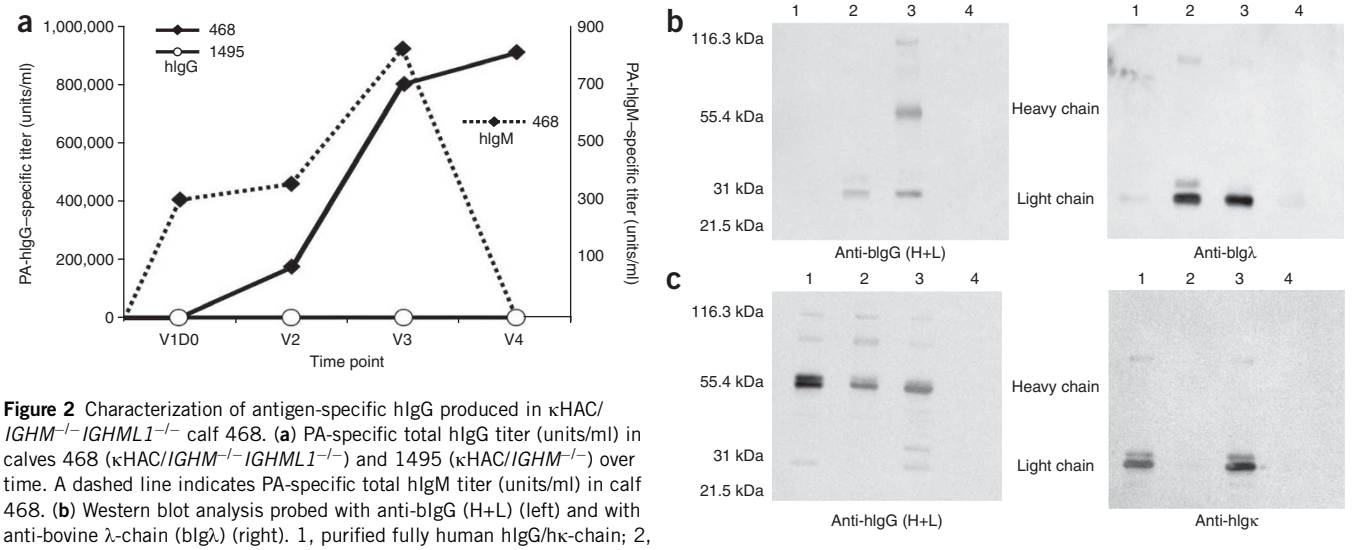

Figure 2 Characterization of antigen-specific hIgG produced in κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ calf 468. (**a**) PA-specific total hIgG titer (units/ml) in calves 468 (κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$) and 1495 (κHAC/*IGHM*$^{-/-}$) over time. A dashed line indicates PA-specific total hIgM titer (units/ml) in calf 468. (**b**) Western blot analysis probed with anti-bIgG (H+L) (left) and with anti-bovine λ-chain (bIgλ) (right). 1, purified fully human hIgG/hκ-chain; 2, purified chimeric hIgG; 3, purified commercial bIgG; 4, purified commercial hIgG. (**c**) Western blot probed with anti-hIgG (H+L) (left) and with anti-human κ-chain (right). 1, purified fully human hIgG/hκ-chain; 2, purified chimeric hIgG; 3, purified commercial hIgG; 4, purified commercial bIgG.

and the hIgM/hIgG ratio was 1.2% on average (**Supplementary Table 1** online). In contrast, hIgG level in κHAC/*IGHM*$^{-/-}$ calves never exceeded ~10 μg/ml. Because the bovine immunoglobulin light chain genes (b*Igl* and b*Igk*) were not inactivated, we also measured the level of fully human hIgG (hIgG/hκ-chain) by a sandwich ELISA. Fully human hIgG/hκ-chain consisted of ~10–20% of total hIgG detected in the serum, which reached levels as high as 649 μg/ml (**Fig. 1c**). The hIgG subclass distribution in calf 468 was similar to that observed in human (**Supplementary Table 2** online).

To evaluate B-cell development in κHAC/*IGHM*$^{-/-}$ and κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$cattle, flow cytometry analysis was performed on cells from Ileal Peyer's patch, the major lymphoid tissue where B-cell development, proliferation and immunoglobulin diversification extensively occur in cattle and other gut-associated lymphoid tissue animals[16,20,22] (**Fig. 1d**). κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ bovines showed improved B-cell development (hIgM^{+}/CD21^{+} mature B cells) compared to κHAC/*IGHM*$^{-/-}$ animals and were comparable to controls. The data suggest that bovine B-cell development can be supported by hIgM in the complete absence of bIgM.

Characterization of antigen-specific hIgG produced in κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ cattle

At the age of 112 d, we immunized calf 468 with anthrax PA[27] to examine the hIgG-mediated humoral immune response (**Fig. 2a**). At vaccination booster 2 (V2), calf 468 began to show a PA–specific hIgG response and reached a high titer at V4. The titer at V4 was higher than the bIgG titer in a control calf 1495 (κHAC/*IGHM*$^{-/-}$) and comparable to PA-specific bIgG titers obtained in wild-type cattle after V16 (**Supplementary Table 3** online). Furthermore, the anti-PA titer obtained from calf 468 was substantially higher than the hIgG anti-PA titer in a human reference serum (AVR801; **Supplementary Table 3**) obtained from donors after four vaccinations with Anthrax Vaccine Adsorbed. On the other hand, as expected, there was no detectable PA-specific hIgG titer in the κHAC/*IGHM*$^{-/-}$ control calf 1495 (**Fig. 2a**). This suggests that the κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ genotype is useful in generating high titer, antigen-specific hIgG after antigen immunization.

For characterization of the PA-specific hIgG produced in cattle, human IgG was purified from the plasma of calf 468 collected after V4 by plasmapheresis. To check the purity of the fully human hIgG/hκ-chain fraction, we performed SDS-PAGE and western blot analysis using anti-bovine IgG (heavy and light chains; H+L) and anti-bovine immunoglobulin λ-chain polyclonal antibodies. There were neither bIgG heavy nor light chain bands detected in the fully human hIgG/hκ-chain fraction (**Fig. 2b**). On the contrary, detection of hIgG heavy and human κ-light chains by anti-hIgG (H+L) polyclonal antibodies and anti-human κ-chain polyclonal antibodies, respectively (**Fig. 2c**), indicates that the fully human hIgG/hκ-chain fraction indeed contains both hIgG heavy and human κ-light chains. We also analyzed human heavy chain and bovine light chain chimeric hIgG obtained from the flow-through fraction of the anti-human κ-chain Sepharose column. The chimeric hIgG fraction was positive for hIgG heavy and bovine light chains, but negative for human κ-light and bIgG heavy chains (**Fig. 2b,c**).

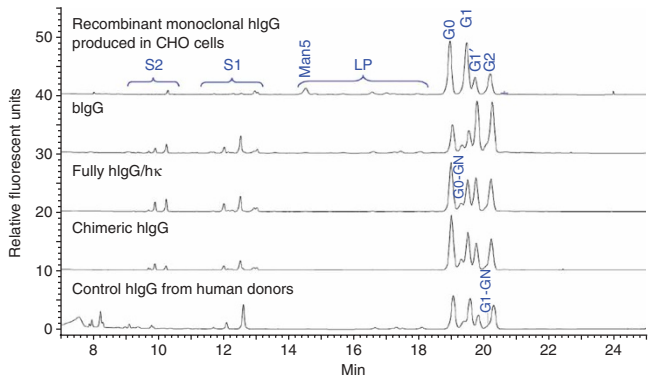

Figure 3 Glycosylation analysis of antigen-specific hIgG produced in κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ calf 468. Capillary gel electrophoresis with helium-cadmium laser–induced fluorescent detection (CE-LIF) on recombinant monoclonal hIgG produced in CHO cells, bIgG from wild-type cattle, fully human hIgG/hκ-chain (hκ) from calf 468, chimeric hIgG from calf 468 and hIgG from human donors (polyglobin-N). S1-S2, monosialyl and bisialyl acids (sialic acid content); LP, mannose and/or afucosylation (fucosylation content); G0, G1, G1′, G2; gal structure (gal content), G0-GN, G1-GN; GlcNAc (GlcNAc content).

Table 2 Toxin neutralization activities

	No. of vaccinations	IgG conc (g/l)	TNA (ED_{50})[a]	TNA (EC_{50})[b] (μg)
Wild-type bovine pooled hyperimmune purified bIgG	16	10.4	10,090	1.0
Calf 468–derived purified total hIgG	4	17.7	12,377	1.4
Calf 468–derived purified chimeric hIgG	4	18.4	13,143	1.4
Calf 468–derived purified fully human hIgG/hκ-chain	4	21.1	11,890	1.8
Human pooled immune serum (AVR 801)	4	5.3	111	57.0

[a]TNA ED_{50} is the dilution of the antibody solution or serum that neutralizes 50% of total cell cytotoxicity by the anthrax toxin. [b]TNA EC_{50} is the amount (μg) of antibody required to neutralize 50% of total cell cytotoxicity by the anthrax toxin.

Furthermore, the percentage of PA-specific IgG fraction was estimated by using a PA-immobilized Sepharose affinity column. Purified bIgG from the control calf 1495 (κHAC/*IGHM*$^{-/-}$), as well as fully human hIgG/hκ-chain and chimeric hIgG from calf 468, were loaded onto the PA affinity column and the PA-specific IgG fraction was eluted at pH 2.5. Compared to control calf 1495, an unusually high proportion of PA-specific antibody, both fully human hIgG/hκ-chain (13%) and chimeric hIgG (35%), was produced by hyperimmunization of calf 468 (**Supplementary Table 4** online).

Glycosylation analysis of antigen-specific hIgG produced in κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ cattle

As the IgG heavy chain is glycosylated at its Fab and Fc regions in a species-specific manner[28], we investigated *N*-linked oligosaccharides both in the fully human hIgG/hκ-chain and chimeric hIgG fractions by capillary gel electrophoresis with helium-cadmium laser–induced fluorescent detection (CE-LIF; **Fig. 3** and **Supplementary Table 5** online). When compared with monoclonal hIgG produced in CHO (Chinese hamster ovary) cells and polyclonal hIgG control from human donors, the glycosylation profile of hIgG (both hIgG/hκ-chain and chimeric hIgG) produced in calf 468 appears to be more similar to that of the polyclonal hIgG control. One minor difference between the bovine-derived hIgG and the control human-derived polyclonal hIgG is in the LP peak, which is thought to contain fucose-less sugar chains. However, the LP peak is similarly minor even in the human control. S1 and S2 peaks contain a sugar chain to which sialic acid is added. The sialic acids, *N*-acetylneuraminic acid (NANA) and *N*-glycolylneuraminic acid (NGNA), were analyzed by reversed-phase high-performance liquid chromatography (HPLC) and fluorescence detection (**Supplementary Table 6** online). Total content of sialic acid is similar between calf 468–derived hIgG and the control human-derived hIgG. However, the ratio of NANA/NGNA is different as expected: calf 468–derived hIgG has predominantly NGNA (similar to the control bIgG[28]), whereas the control polyclonal hIgG exclusively has NANA. With respect to branched sugar chains (G0-G2), the contents of galactose (galactose residue per *N*-glycan) and *N*-acetylglucoseamine (GlcNAc) (G0-GN and G1-GN) are similar between the calf 468–derived hIgG and the control human-derived hIgG.

TNA and mouse protection assay of PA-specific hIgG produced in κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ cattle

The purified fully human hIgG/hκ-chain and chimeric hIgG fractions containing the binding activity against PA antigen at V4 were evaluated by the TNA[29,30] (**Table 2**). The TNA of hIgG produced in calf 468 (both purified fully human hIgG/hκ-chain and chimeric hIgG fractions) is comparable to that of hyperimmunized wild-type bIgG and much higher than that of the human reference. Our PA-challenge mouse protection assay (**Fig. 4**) involved challenging mice with 1×10^6 anthrax (Sterne strain) spores. Mice were given either 90 mg/kg of total hIgG produced in calf 468 at V1 (contained little activity); 90 mg/kg of fully human hIgG/hκ-chain or 70 mg/kg of chimeric hIgG or 70 mg/kg of total hIgG (hIgG/hκ-chain + chimeric hIgG) from calf 468 at V4; or 50 mg/kg of hyperimmunized pooled wild-type bIgG at V16. IgG doses were standardized to contain equivalent TNA activity in the purified fraction. With the negative control (bovine-derived hIgG at V1), nine out of ten mice died, whereas both fully human hIgG/hκ-chain and chimeric hIgG collected from calf 468 at V4 completely protected all ten mice. The hyperimmune pooled bIgG resulted in the death of one of the ten mice tested. This complete protection activity was also observed with 22.5 mg/kg and 17.5 mg/kg of fully human hIgG/hκ-chain and chimeric hIgG, respectively, from calf 468 at V4 (**Fig. 4**). These data suggest that hIgG produced in calf 468 (both fully human hIgG/hκ-chain and chimeric hIgG) was fully functional and effective in neutralizing the toxin activity *in vitro* and *in vivo*.

DISCUSSION

This study demonstrates the feasibility of producing a large quantity of highly active, antigen-specific hpAbs in a large farm animal species. Calf 468 produced over 2 g/l of total serum hIgG (fully human and chimeric). Moreover, we showed that hyper-immunization with a PA antigen resulted in high *in vitro* and *in vivo* neutralization potency. The high activity may be attributed to an unusually high percentage of PA-specific, fully human and chimeric hIgG. In the human reference serum AVR801, the percentage of PA-specific hIgG is estimated to be 2.1%[31]. The high antigen specificity should be beneficial for therapeutic applications.

To generate a Tc calf capable of producing a large volume of functional hIgG, several difficult challenges were addressed. We have shown that, unlike mouse and human, cattle have two independent

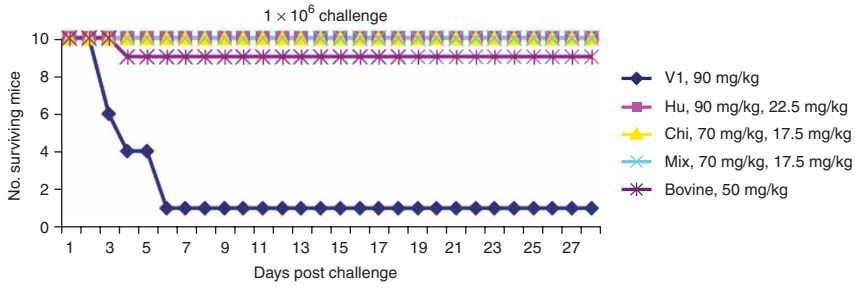

Figure 4 *In vivo* mouse protection assay of PA-specific hIgG produced in κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ calf 468. V1, purified total hIgG from calf 468 at V1 of PA-immunization; Hu, purified fully human hIgG/hκ-chain from calf 468 at V4 of PA-immunization; Chi, purified chimeric hIgG from calf 468 at V4 of PA-immunization; Mix, purified total hIgG from calf 468 at V4 of PA-immunization; Bovine, hyperimmunized pooled wild-type bIgG at V16 of PA-immunization.

pathways for B-cell development regulated by the two distinct fully functional IgM heavy chain loci, *IGHM* and *IGHML1*, and that the inactivation of both these loci is critical for producing large quantities of hIgG. This is the first demonstration of a mammalian species that has multiple fully functional *IgH* loci. As other ungulates, such as sheep and goat, may also possess a similar *IGHML1* locus in addition to the classical *IGHM* gene[10], the double-knockout approach of the two IgM loci may be equally useful for production of hpAbs in other large farm animals.

Another challenge was to produce a viable calf following four gene-targeting events and insertion of a HAC: a total of five sequential genetic modifications and six cloning procedures. Calf 468 was actually produced from a seventh cloning procedure. As additional κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ calves have comparably high levels of serum hIgG (**Supplementary Table 7** online), the κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ genotype appears to be useful for producing a large quantity of hIgG. However, the low rate of development to term and relatively high incidence of mortality after birth are impediments for commercial production. Results presented in several studies show dramatic declines in the efficiency of cloning with successive cloning procedures[32–36]. One possible reason for the decrease in efficiency is the accumulation of epigenetic errors, including imprinting errors, induced by embryonic cloning. To solve this potential problem, we have incorporated a plan to produce IgM double-knockout (*IGHM*$^{-/-}$*IGHML1*$^{-/-}$) cell lines by mating. Our preliminary results of breeding between highly recloned male (*IGHM*$^{-/+}$*IGHML1*$^{-/-}$) and female (*IGHM*$^{-/+}$*IGHML1*$^{-/+}$) parents indicate that the rate of development to term of calves and of survival after birth is improved to a level similar to that of calves derived from *in vitro* fertilization.

Stability of κHAC was examined from both mitotic and structural perspectives. The former was done by fluorescent *in situ* hybridization (FISH) analysis using human COT1 DNA as a probe. As >90% of cells observed generally retain κHAC as a single copy–independent chromosome both in peripheral blood lymphocytes and fibroblasts for at least several years, κHAC appears to be mitotically stable during development. We tested structural stability by genomic PCR mapping with 16 markers dispersed over the entire HAC structure. Most of the animals tested were positive for all the markers (13 out of 15 animals), with the exception of two calves which were missing some markers. Overall, κHAC is retained at a high rate and with high fidelity during development.

In the current Tc bovine system, ~80% of total serum hIgG produced is chimeric; consisting of human IgG heavy and bovine immunoglobulin light chains. As the chimeric hIgG is fully functional and is likely not highly immunogenic, a mixture of chimeric and fully human hIgG could be safer and more useful than fully animal-derived polyclonal antibodies, for single, or minimal repetitive, dose treatments. However, fully human hIgG would be preferred for applications that require long-term, repetitive treatments. Fully human hIgG could be derived by purification, as demonstrated in this study. Notably, the serum level of fully human hIgG/hκ-chain in calf 468 was ~500 μg/ml in spite of the presence of the bovine immunoglobulin light chain genes. This level is comparable to that of hIgG-producing transgenic mice in which both murine *Igh* and *Igk* genes are knocked out[8]. The human IgG heavy chain may preferentially pair with human κ-light chain, rather than with bovine immunoglobulin light chain. However, knocking out the bovine immunoglobulin light chain genes would be preferable for higher yields of fully human hIgG.

In the present type of genetic modification (κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$), other classes of chimeric IgG heavy chain—for example, trans-class switched or trans-spliced IgG heavy chain—could be generated[37,38]. Because the bovine Cγ region is still intact in the *IGHM*$^{-/-}$*IGHML1*$^{-/-}$ double knockout, a heavy chain comprising human $V_HD_HJ_H$ and bovine Cγ sequences could be produced. To investigate this possibility, we performed RT-PCR with one primer located in human V_H and the other in bovine Cγ sequence from two κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ newborn calves. We detected human $V_HD_HJ_H$ and bovine Cγ-comprising transcript in the sample from one animal (**Supplementary Fig. 3** online) and the result was confirmed by sequencing. Another issue concerning the *IGHM*$^{-/-}$*IGHML1*$^{-/-}$ double knockout is the possibility that bovine $V_HD_HJ_H$ and bovine Cγ-comprising transcripts could be generated from an in *cis* class switch mechanism on the bovine *IgH* locus once hIgM$^+$ B cells are activated. To investigate this possibility, we conducted RT-PCR to amplify $V_HD_HJ_H$-rearranged bovine *IGHG* transcripts from two animals and detected bovine *IGHG* transcripts at low levels, with confirmation by sequence analysis (**Supplementary Fig. 3** online). Both chimeric heavy chain and fully bovine bIgG heavy chain are removed by our purification process and are not detected after purification (**Fig. 2b**).

It has been suggested that cattle can use gene conversion for immunoglobulin gene diversification[19–22]. Gene conversion might cause small segments of bovine V (or pseudo V) sequence to be placed into the human V sequence. We investigated this possibility using RT-PCR to amplify human $V_HD_HJ_H$-rearranged human Cγ transcripts from four κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ animals (primers used in this RT-PCR also amplify bovine sequence). The RT-PCR products were subcloned for sequence analysis (31 subclones were analyzed). Excluding the CDR3 region (D_H segment), sequence analysis showed >90% homology with human sequence (V_H and J_H) and no obvious trace of bovine sequence was detected.

Both polyclonal antibodies collected from human plasma donors and monoclonal antibodies produced by fermentation have been extraordinarily beneficial for treating a wide variety of human diseases. Our Tc bovine system for production of hpAbs may help to expand the repertoire of diseases that can be successfully treated using antibody-based therapeutics.

METHODS

All animal procedures were performed in compliance with Hematech's guidelines, and protocols were approved by the Institutional Animal Care and Use Committee.

Construction of genomic library and library screening. Genomic DNA was extracted from the *IGHML1*$^{-/-}$ fibroblast cell line 4658, originally derived from a primary bovine fibroblast line 6939 and a λ-phage-based genomic library was constructed using λFIX II vector through a custom library construction service (Lofstrand). A PCR product amplified with a primer pair (bCμf2 × bCμr2) was ^{32}P-labeled using Rediprime II DNA Labeling System kit (Amersham Biosciences) according to the manufacturer's manual, to use as a probe. This probe was able to hybridize to exon 2-3 of both *IGHM* and *IGHML1* genes. Plaque hybridization was carried out under a standard protocol. Positive phage plaques hybridized with the probe were propagated and DNA was extracted and purified using Wizard Lambda Preps DNA Purification System kit (Promega) according to the manufacturer's manual. The phage clones were classified into four alleles based on sequence identity. Alleles *U* and *u* contained the *puro* and *neo* STOP knockout cassettes[23], and essentially matched the sequence of the *IGHML1* locus (U63637 and AY149283) as expected. Alleles *AY* and *ay* were intact and matched the sequence of the *IGHM* locus (AY230207 and AY158087).

Construction of targeting vectors. The 7.5 kb of *Sal*I-*Bgl*II genomic fragment (5′ homologous arm) and 2.0 kb of *Bgl*II-*Bam*HI fragment (3′ homologous arm) around the exon 2 of alleles *ay* and *AY* of *IGHM* gene were subcloned into

pBluescript II SK(–) (Stratagene), and then *hyg* or *bsr*, STOP cassette (Stratagene) and DT-A (diphtheria toxin A) genes were inserted (pbCμayKOhyg vector and pbCμAYKObsr vectors, respectively), as previously described[23]. For ploxPHygcos138 (F), genomic sequence of *cos138* was amplified with a primer; cos138-F6B × cos138-R6B, and cloned to the *Bam*HI site in pBluescript II SK(–). Hyg-PGK-*loxP* cassette[26] was cloned to the *Spe*I site in the *cos138* genomic sequence, followed by DT-A subcloning. Primer sequences: cos138-F6B (5′-TCGAGGATCCCACATAGACATTCAACCGCAAAGCAG-3′), cos138-R6B (5′-TCGAGGATCCAGGCCCTACACATCAAAAAGTGAAGCAG-3).

Construction of κHAC vector. κHAC vector was constructed using a previously described chromosome-cloning system[6,26]. Briefly, a DT40 clone, containing a hChr2 fragment truncated at the *CD8A* locus, was electroporated (550 V, 25 μF) with ploxPHygcos138 (F) targeting vector (25 μg) to integrate a *loxP* sequence at the *cos138* locus. Colonies were selected by hygromycin B (1.5 mg/ml) for 2 weeks and their DNA was subjected to PCR screening with cos138KO-F × cos138KO-R primers under the following conditions: 98 °C for 10 s, and 65 °C for 8 min in 40 cycles. A clone κTL1 was identified and fused to a DT40 clone (R56) containing the stable and germline-transmittable human microchromosome vector, SC20. The SC20 vector contained a *loxP* sequence integrated at the *RNR2* locus[26]. The resulting DT40 hybrids contained the two human chromosome fragments. The DT40 hybrid clone (κ1R) was then transfected with a Cre recombinase-expression vector to induce Cre/*loxP*-mediated chromosomal translocation between the hChr2 fragment and the SC20 vector. The stable transfectants were analyzed using nested PCR[26] to confirm the occurrence of chromosomal translocation. FISH analysis and fluorescent-activated cell sorting (FACS) of green fluorescent protein–expressing cells[26] were also used to confirm the presence of κHAC. Primer sequences: cos138KO-F (5′-TCTTTCTCTCACCTAATTGTCCTGGC-3′), cos138KO-R (5′-AGGACTGGCACTCTTGTCGATACC-3′).

Genetic modification of bovine fibroblasts. Bovine fetal fibroblasts were cultured and transfected as previously described[23]. Briefly, fibroblasts were electroporated with 30 μg of pbCμayKOhyg or pbCμAYKObsr vector at 550 V and 50 μF. After 48 h, the cells were selected under 200 μg/ml of hygromycin B or 10 μg/ml of blasticidine-HCl for 2 weeks and resistant colonies were picked up and transferred to replica plates; one was for genomic DNA extraction and the other was for embryonic cloning. Microcell-mediated chromosome transfer was done with the κHAC vector as described previously[6].

Genomic PCR analyses. Genomic DNA was extracted from the replica 24-well plates, fetuses or ear biopsies from calves, using a Puregene DNA extraction kit (GentraSystem). For genotyping *IGHML1*$^{-/-}$, primer pairs PuroF2 × PuroR2 and NeoF3 × NeoR3 were used as described previously[23]. To identify heterozygous *IGHM*$^{-/+}$ genotype, primer pair ayKOhygF2 × ayKOhygR2 was used. Forty cycles of PCR were performed by incubating the reaction mixtures in the following conditions: 98 °C for 10 s, and 68 °C for 8 min. To identify homozygous *IGHM*$^{-/-}$ genotype, primer pair AYKObsrF2 x AYKObsrR2 was used, together with ayKOhygF2 × ayKOhygR2 primers, as above. For genotyping *IGHM*$^{-/-}$*IGHML1*$^{-/-}$, all the four primer pairs ayKOhygF2 × ayKOhygR2, AYKObsrF2 × AYKObsrR2, PuroF2 × PuroR2 and NeoF3 × NeoR3 were used. All the PCR products were run on 0.8% agarose gels. Primer sequences: ayKOhygF2 (5′-TGGTTGGCTTGTATGGAGCAGCAGAC-3′), ayKOhygR2 (5′-TAGGATATGCAGCACACAGGAGTGTGG-3′), AYKObsrF2 (5′-GGTAGTGCAGTTTCGAATGGACAAAAGG-3′), AYKObsrR2 (5′-TCAGGATTTGCAGCACACAGGAGTG-3′), PuroF2 (5′-GAGCTGCAAGAACTCTTCCTCACGC-3′), PuroR2 (5′-ATGTACCTCCCAGCTGAGACAGAGGG-3′), NeoF3 (5′-TTTGGTCCTGTAGTTTGCTAACACACCC-3′), NeoR3 (5′-GGATCAGTGCCTATCACTCCAGGTTG-3′). In addition, Southern hybridization using each of the drug-resistant genes as a probe was performed to confirm a single-site integration of each of the knockout cassettes.

RT-PCR analysis. RNA was extracted from spleens of fetuses or peripheral blood mononuclear cells from calves using an RNeasy mini kit (Qiagen) and first-strand cDNA synthesis was done using the superscript first strand synthesis system for RT-PCR (Invitrogen). PCR was done using primer pairs; mBCμF2 × mBCμR2 (**Supplementary Fig. 1c**), BL17 (located in the leader exon of bovine immunoglobulin heavy chain) × mBCμR2 and BL17 × bCγ1R2 in 40 cycles composed of 98 °C for 10 s, 62 °C for 30 s, 72 °C for 1 min. For detection of bovine *β-actin* mRNA expression, bBAF and bBAR primers were used in the same PCR condition. To exclude the possibility of genomic DNA contamination, another RT-PCR was performed without reverse transcriptase. The PCR products were run on 0.8% agarose gel. Primer sequences: mBCμF2 (5′-GCATGCTGACCATCACAGAG-3′), mBCμR2 (5′-GTTCAGGCCATCATAGGAGG-3′), BL17 (5′-CCCTCCTCTTTGTGCTGTCA-3′), bCγ1R2 (5′-GGGAGCTCAGGGGGTGGGCAACAGTCA-3′), bBAF (5′-ACATCCGCAAGGACCTCTAC-3′), bBAR (5′-AACCGACTGCTGTCACCTTC-3′).

Flow cytometry analysis. Peripheral blood was collected from 180-d-old fetuses or calves by jugular venipuncture into heparinized tubes. Ileum and cecum were also collected in AIMV cell culture medium (Invitrogen-GIBCO). Whole white blood cells (leukocytes) were isolated from heparinized blood using RBC-lysis buffer (Sigma). Lymphocytes from Ileal Peyer's patch were isolated by mechanical disruption and filtered using a 40 μm nylon cell strainer (BD Biosciences) before density-centrifugation using Ficoll-Paque PLUS (GE Healthcare Biosciences). Sheep anti-bovine IgM-biotin (Bethyl) and F(ab′)$_2$ goat anti-human IgM-biotin (Serotec) followed by streptavadin-PE-Cy5 (Caltag) were used to label surface IgM on the B cells. To label surface B220 marker on developing bovine B cells, we used mouse anti-bovine B220 (CD45R) antibody clone GS5A (VMRD) followed by anti-mouse IgG1-PE secondary antibody (Caltag). Mouse anti-bovine CD21 Clone MCA1424 (Serotec) directly labeled with PE was used to detect surface CD21 marker on bovine B cells. Staining was done by a standard protocol and then analyzed by FACScan or FACSAria flow cytometer (BD Biosciences).

Western blot. Immunoglobulin heavy and light chains were separated by SDS PAGE using 4–12% precast Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride membranes that were directly probed with specific horseradish peroxidase (HRP)-conjugated antibodies following blocking. The HRP-conjugated antibodies were: goat anti-bIgG (heavy and light; H+L) HRP (KPL) for bIgG heavy chain, goat anti-bIgG (Fab′)$_2$ HRP (Jackson ImmunoResearch) for bovine light chain, donkey anti-hIgG (H+L) HRP (Jackson ImmunoResearch) for hIgG heavy chain, and goat anti-hIgκ light chain HRP (Bethyl) for human κ-light chain. All HRP-conjugated antibodies for bovine and human IgGs were confirmed to have no species cross-reactivity.

ELISA. ELISA assays were sandwich type using an affinity-purified capture antibody and an appropriate HRP-enzyme–labeled detection antibody. For bIgM detection, sheep anti-bIgM affinity-purified (Bethyl) as a capture and sheep anti-bIgM-HRP as a detection antibody were used. For bIgG detection, sheep anti-bIgG affinity-purified as a capture and sheep anti-bIgG-HRP as a detection antibody were used. Detection was performed by a standard protocol.

hIgG was analyzed by using a commercial ELISA test (Bethyl). All assay steps were carried out as per manufacturer. Briefly, human reference serum (Standard) supplied in the kit was diluted to 500 ng/ml and then to 7.8 ng/ml in 1:2 serial dilutions (total of seven dilutions) in PBS/0.1% Tween 20 (PBS/Tween). Nunc Maxisopr Immuno plates were coated with affinity-purified goat anti-hIgG capture antibody at 10 μg/ml concentration, 100 μl/well at 25 °C for 1.5 h. Plates were washed three times with 200 μl of PBS/Tween buffer using a plate washer. Standards were loaded (500 ng/ml to 7.8 ng/ml) at 100 μl/well in duplicate wells. Four 1:2 serial dilutions of each serum samples were loaded in duplicates at 100 μl/well. Plates were covered and incubated at 25 °C for 1 h. Plates were then washed three times with PBS/Tween as described earlier. Sheep anti-hIgG HRP-conjugate antibody was diluted 1:100,000 in PBS/Tween and loaded at 100 μl/well for all wells. Plates were then incubated for 1 h at 25 °C and washed again three times with PBS/Tween. 1:1 mix of TMB/H_2O_2 substrate system (KPL) was added at 100 μl/well and color development was allowed for 20–25 min. Color reaction was stopped by adding 100 μl/well of 10% phosphoric acid Stop reagent and plates were read in a microplate reader at 450 nm. A standard curve (log to linear) was drawn with OD_{450} reading on the *y* axis and $\log_{10}$ concentrations (ng/ml) on *x* axis and average sample readings were interpolated in the graph to obtain ng/ml concentrations of each sample, using an automated Excel worksheet module. Final μg/ml concentration of hIgG was calculated by taking the mean of all dilutions of each sample in the linear portion of the curve.

OVA-immunization. *IGHM*$^{-/-}$, *IGHML1*$^{-/-}$ and *IGHM*$^{-/-}$*IGHML1*$^{-/-}$ calves and control wild-type calves were immunized with Ovalbumin (OVA) antigen (Sigma) at 1 mg/dose formulated with Montanide ISA 25 adjuvant (Seppic) as water-in-oil emulsion. The calves were immunized three times at 3-week intervals (primary immunization followed by first booster after 3 weeks and second booster after 6 weeks). Vaccine was administered by intramuscular injection (2 ml dose containing 1 mg/ml OVA plus 1 ml of ISA-25 adjuvant) in the neck region. Serum samples were collected before each immunization (V1, V2 and V3) and 7 d and 14 d after each immunization for antibody titer analysis. Blood was drawn into serum separator tubes, allowed to clot and serum was separated by centrifugation. Serum was then aliquoted in 0.5–1 ml volumes and stored frozen until assays were performed. Anti-OVA antibody titers were determined by OVA-specific IgG ELISA.

IBR-immunization. *IGHM*$^{-/-}$ calves and wild-type control calves were immunized with Triangle 4, which contained IBR antigen (Fort Dodge Animal Health). Vaccine was administered by subcutaneous injection in the neck region at 2 ml per dose. The animals were boosted four more times, with an interval of 3 weeks for each booster for the first to fourth vaccinations, and an interval of 6 weeks between the fourth and fifth vaccinations. Serum samples were taken right before each immunization (V1 to V4) and 7 d and 14 d after each immunization for antibody titer analysis. Blood was drawn into serum separator tubes (tiger-top), allowed to clot and serum was separated by centrifugation. Serum was then aliquoted in 0.5–1 ml volumes and stored frozen until assays were performed. Anti-IBR antibody titers were determined by IBR-specific IgG ELISA with a commercial bovine rhinotrachetis virus antibody test kit (IDEXX).

PA-immunization. κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ calves and κHAC/*IGHM*$^{-/-}$ control calves were immunized with anthrax recombinant protective antigen (rPA) antigen (List Biological) at 2 mg/dose formulated with Montanide ISA 206 adjuvant (Seppic) as a water-in-oil-in-water emulsion. The calves were immunized four times with 4-week intervals. Vaccine was administered by intramuscular injection (2 ml per dose containing 2 mg/ml PA plus 1 ml of ISA-206 adjuvant) in the neck region. Serum samples were collected before each immunization (V1 to V4) and 7 d, 10 d and 14 d after each immunization for antibody titer analysis. Blood was drawn into serum separator tubes, allowed to clot and serum was separated by centrifugation. Serum was then aliquoted in 0.5–1 ml volumes and stored frozen until assays were performed. Anti-PA antibody titers were determined by PA-specific IgG ELISA as follows.

To determine PA-specific hIgG titers, 96-well Immuno 2-HB ELISA plates were coated by adding 100 µl per well of 2 µg/ml of rPA (List Biological) in PBS at pH 7.4 and incubating overnight (12–16 h) at 4 °C. rPA-coated plates were then washed three times with 200 µl of PBS/0.05% Tween 20. Serum samples were diluted in PBS/0.05% Tween 20 buffer with 5% membrane blocking agent (non-fat dry milk) in four serial dilutions. High-titer purified hIgG from calf 468 with a predetermined end-point titer was used as the standard and seven 1:3 serial dilutions from 1:9,000 to 1:6,561,000 were prepared in PBS/0.05% Tween 20 buffer for the standard curve. Reciprocal of the end-point dilution was used as titer units, and for the standard, the end-point titer was determined and assigned as 7,400,000 units. A positive-control serum with predetermined titer (900,000 units) and a negative-control serum with no titer were also diluted serially in PBS/0.05% Tween 20 buffer with non-fat dry milk and were used as internal controls to monitor consistency of the assays. The calibrator standard serum dilutions, positive-control serum, negative-control serum and test serum samples were added in duplicate wells at 100 µl/well in rPA-coated plates and incubated for 1 h at 37 °C. Plates were washed three times with PBS/0.05% Tween 20 buffer to remove unbound proteins and 100 µl of donkey anti-hIgG-HRP–labeled antibody (Jackson Immuno Research) diluted at 1:50,000 in PBS/T buffer with non-fat dry milk added to each well. Plates were incubated for 1 h at 37 °C and washed three times with in PBS/0.05% Tween 20. Finally, the bound anti-PA antibodies were detected by adding 100 µl/well TMB +H_2O_2 substrate mix (KPL) and incubated for 10 min at 25 °C. The reaction was stopped by adding 100 µl 10% phosphoric acid and read in Microplate Reader (Biotek Instruments) at 450 nm. A four-parameter standard curve was generated using seven serial dilution values and serum sample values were calculated by interpolation on the curve by KC-4 software. Average titer values from three or four test dilutions were calculated for each test serum sample. Similarly, PA-specific bIgG titers were determined.

TNA assay. The TNA assay was performed as described previously[37] with some modifications. In brief, cells were plated in a 96-well assay plate and allowed to adhere overnight in a 37 °C, 5% CO_2 incubator so that they would reach a density of 40–60% confluency the following morning. Sera from calves that had been vaccinated with rPA as described above was prepared in a twofold sequential dilution and distributed into a separate 96-well plate. A fixed dose of lethal toxin (a mixture of rPA and rLF) was added to each of the serum dilutions and the mixtures were incubated for 1 h in a 37 °C, 5% CO_2 incubator. The lethal toxin/serum mixtures were then added to the cells in the individual wells of the 96-well plate and incubated for 4 h. This 4-h incubation provides the time for any remaining active lethal toxin to lyse the cells. Cells were washed, stained with thiazol blue (MTT; Sigma) and incubated for 1 h at 37 °C. To determine the cell viability, we plotted OD_{570} readings (with background subtracted out) against the dilutions of the serum samples. This analysis allows for the calculation of either an end-point titer or an effective-dose 50% (ED_{50}), which is the dilution of sera in which one-half of the lethal toxin is neutralized.

Mouse protection assay. Groups of ten female A/J mice (Jackson Laboratories) at ~7 weeks of age were challenged with the Sterne strain of anthrax spores (Colorado Serum). Spores were administered at a dose of 1×10^6 spores by intraperitoneal (IP) injection. Spores had been prepared by washing three times in sterile water to remove the saponin that is present in the commercial preparation. Washed spores were stored in sterile water and the titer of spores was determined on nutrient agar plates. Spores were diluted with sterile water so that the appropriate dose per mouse was in a 200 µl volume. Mice were treated with purified IgG preparations at 4 h after challenge. Total purified hIgG from calf 468 contained both fully human and chimeric hIgG molecules. Mice received total hIgG, fully human hIgG, chimeric hIgG, pre-immune total hIgG (negative control) or a pooled bIgG positive control. All antibody treatments were administered by IP injection in a 200 µl volume. Mice were observed twice daily for 28 d and moribund animals were euthanized.

Purification of fully human hIgG/hκ-chain and chimeric hIgG fractions. Plasma bags were thawed at 25 °C overnight and total protein concentration was determined. One volume of purified water was added to the plasma, followed by adjusting to pH 4.8 with 20% acetic acid. Caprylic acid was slowly added to the sample (with continuous mixing) to a final concentration of 6.0%. The sample was mixed for 30 min and filtered using a depth filter device. The filtrate was adjusted to neutral pH and loaded onto an anti-hIgG Fc affinity column (6CP Sepharose) equilibrated with PBS. The column was eluted with pH 3 solution to recover IgG. The 6CP column elution peak was neutralized and then passed through an anti-bIgG Fc column (HC15 Sepharose) to remove residual bIgG. To separate fully human hIgG from chimeric hIgG, the IgG sample was applied onto an anti-human F(ab′)$_2$ κ Sepharose column. The flow-through fraction contained chimeric hIgG, whereas the pH 3.0 eluted peak was fully human hIgG. Samples were then dialyzed into PBS and stored at 2–8 °C.

Glycosylation analysis. *N*-linked oligosaccharide profiling was done as follows. A sample of antibody (0.5 mg) was diluted with water (total 49 µl) in a sample tube (1.5 ml). 2-mercaptoethanol (1 µl) and PNGase F (10 units, 10 µl) were added to the mixture and incubated at 37 °C for 20–24 h. After addition of ethanol (150 µl), the mixture was centrifuged at 15,000*g* for 15 min. The supernatant containing the released oligosaccharides was transferred to a new sample tube and evaporated to dryness. *N*-linked oligosaccharides in the mixture were labeled with 2-aminobenzoic acid (2-AA) according to the method reported previously[39]. Briefly, water (20 µl) was added to the dried oligosaccharide sample. A derivatization reagent was freshly prepared by dissolution of 2-AA and sodium cyanoborohydride (30 mg and 20 mg, respectively) in methanol (1 ml) containing 4% sodium acetate and 2% boric acid. This reagent (100 µl) was then added to the oligosaccharide solution. The mixture was kept at 80 °C for 1 h. After cooling followed by addition of water (30 µl), the oligosaccharide mixture was purified using a solid-phase extraction

column (Oasis HLB cartridges, 1 ml, Waters). The reaction solution was diluted with 1.0 ml of acetonitrile-water (95:5), mixed vigorously and applied to a cartridge previously equilibrated with the same solvent (1 ml × 2). After washing the cartridge with acetonitrile-water (95:5, 1 ml × 2), the fluorescence-labeled oligosaccharides were eluted with acetonitrile-water (20:80, 1 ml) and the eluate was evaporated to dryness by a centrifugal evaporator. The residue was dissolved in water (100 µl), and a portion (typically 5 µl) was used for the analysis by CE-LIF. Capillary electrophoresis was performed on a ProteomeLab PA800 system (Beckman Coulter) equipped with a helium-cadmium laser–induced fluorescence detector (excitation 325 nm, emission 405 nm) using a DB-1 capillary (100 µm internal diameter, 30 cm effective length, 40 cm total length, Agilent/J&D Scientific) in 100 mM Tris-borate buffer (pH 8.3) containing 10% PEG35000 as the running buffer. PEG was added to diminish electroendoosmotic flow and improve the resolution. For pressure injection, sample solutions were introduced into the capillary at 1 p.s.i. for 10 s. Separation was performed by applying 25 kV at 25 °C at reverse polarity.

Sialic acid content analysis was carried out as follows. A sample of antibody (0.4 mg) was diluted with water (total 100 µl) in a sample tube (1.5 ml). Hydrolysis solution (water/acetic acid; 27:8; 100 µl) was added to the sample, and incubated at 80 °C for 2.5 h. Then 1,2-diamino-4,5-methylenedioxybenzene solution (200 µl) was added and the mixture was kept at 60 °C for 2 h in the dark. After cooling, 1 M NaOH (200 µl) was added to stop the reaction. The derivatized sialic acids were separated by reversed phase HPLC using a C18 column (9 × 150 mm, Symmetry, Waters) and mobile phase (water/acetonitrile/methanol; 84:9:7) at 0.6 ml/min. Detection was performed using fluorescence detector (excitation 373 nm, emission 448 nm). Sialic acid content was calculated from a standard curve generated from known concentrations of NANA and NGNA derivatized in a same manner as the sample.

Embryonic cloning. Cloned fetuses and calves were produced using chromatin transfer procedure as described previously[6,23]. Both *IGHM*$^{-/-}$*IGHML1*$^{-/-}$ and κHAC/*IGHM*$^{-/-}$*IGHML1*$^{-/-}$ calves were maintained with ~7 mg/ml of exogenous bIgG supplied as bovine intravenous immunoglobulin from wild-type cattle donors.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

Y.K. and J.M.R. led the work and wrote the manuscript. P.K. and J.K. led animal cloning. T.S. and H.W. led immunological analyses and immunization. J.J. led purification and protein chemistry. H.M. and J.S. carried out gene targeting experiments. J.M. conducted the mouse challenge assay. M.H. performed flow cytometry analysis. S.K. and K.T. implemented sugar chain analyses. I.I. initiated the work.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/

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1. Lemieux, R., Bazin, R. & Neron, S. Therapeutic intravenous immunoglobulins. *Mol. Immunol.* **42**, 839–848 (2005).
2. Jolles, S., Sewell, W.A.C. & Misbah, S.A. Clinical uses of intravenous immunoglobulin. *Clin. Exp. Immunol.* **142**, 1–11 (2005).
3. Newcombe, C. & Newcombe, A.R. Antibody production: polyclonal-derived biotherapeutics. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **848**, 2–7 (2007).
4. Lonberg, N. Human antibodies from transgenic animals. *Nat. Biotechnol.* **23**, 1117–1125 (2005).
5. Waltz, E. Polyclonal antibodies step out of the shadows. *Nat. Biotechnol.* **24**, 1181 (2006).
6. Kuroiwa, Y. *et al.* Cloned transchromosomic calves producing human immunoglobulin. *Nat. Biotechnol.* **20**, 889–894 (2002).
7. Kitamura, D., Roes, J., Kuhn, R. & Rajewsky, K.A. B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin µ chain gene. *Nature* **350**, 423–426 (1991).
8. Tomizuka, K. *et al.* Double trans-chromosomic mice: Maintenance of two individual human chromosome fragments containing Ig heavy and κ loci and expression of fully human antibodies. *Proc. Natl. Acad. Sci. USA* **97**, 722–727 (2000).
9. Yel, L. *et al.* Mutations in the mu heavy chain gene in patients with agammaglobulinemia. *N. Engl. J. Med.* **335**, 1486–1493 (1996).
10. Hayes, H.C. & Petit, J.E. Mapping of the β-lactoglobulin gene and of immunoglobulin M heavy chain-like sequence to homologous cattle, sheep and goat chromosomes. *Mamm. Genome* **4**, 207–210 (1993).
11. Hosseini, A., Campbell, G., Prorocic, M. & Aitken, R. Duplicated copies of the bovine J_H locus contribute to the Ig repertoire. *Int. Immunol.* **16**, 843–852 (2004).
12. Chowdhary, B.P., Fronicke, L., Gustavsson, I. & Scherthan, H. Comparative analysis of the cattle and human genomes: detection of ZOO-FISH and gene mapping-based chromosomal homologies. *Mamm. Genome* **7**, 297–302 (1996).
13. Tobin-Janzen, T.C. & Womack, J.E. Comparative mapping of *IGHG1, IGHM, FES* and *FOS* in domestic cattle. *Immunogenetics* **36**, 157–165 (1992).
14. Gu, F., Chowdhary, B.P., Andersson, L., Harbitz, I. & Gustavsson, I. Assignment of the bovine immunoglobulin gamma heavy chain (IGHG) gene to chromosome 21q24 by in situ hybridization. *Hereditas* **117**, 237–240 (1992).
15. Zhao, Y., Kacskovics, I., Rabbani, H. & Hammarstrom, L. Physical mapping of the bovine immunoglobulin heavy chain constant region gene locus. *J. Biol. Chem.* **278**, 35024–35032 (2003).
16. Reynaud, C.A., Mackay, C.R., Muller, R.G. & Weill, J.-C. Somaticgeneration of diversity in a mammalian primary lymphoid organ: the sheep ileal Peyer's patches. *Cell* **64**, 995–1005 (1991).
17. Jenne, C.N., Kennedy, L.J., McCullagh, P. & Reynolds, J.D. A new model of sheep Ig diversification: shifting the emphasis toward combinatorial mechanisms and away from hypermutation. *J. Immunol.* **170**, 3739–3750 (2003).
18. Butler, J.E. Immunoglobulin diversity, B-cell and antibody repertoire development in large farm animals. *Rev. Sci. Tech.* **17**, 43–70 (1998).
19. Butler, J.E. Immunological diversity, B-cell and antibody repertoire development in large farm animals. *Rev. Sci. Tech.* **17**, 43–70 (1998).
20. Meyer, A. *et al.* Immunoglobulin gene diversification in cattle. *Int. Rev. Immunol.* **15**, 165–183 (1997).
21. Aitken, R. *et al.* Structure and diversification of the bovine immunoglobulin repertoire. *Vet. Immunol. Immunopathol.* **72**, 21–29 (1999).
22. Lucier, M.R. *et al.* Multiple sites of Vλ diversification in cattle. *J. Immunol.* **161**, 5438–5444 (1998).
23. Kuroiwa, Y. *et al.* Sequential targeting of the genes encoding immunoglobulin-µ and prion protein in cattle. *Nat. Genet.* **36**, 775–780 (2004).
24. Alt, F.W., Blackwell, T.K. & Yancopoulos, G.D. Development of the primary antibody repertoire. *Science* **238**, 1079–1087 (1987).
25. Hood, L., Gray, W.R., Sanders, B.G. & Dreyer, W.Y. Light chain evolution: antibodies. *Cold Spring Harbor Symp. Quan. Bio.* **32**, 133–146 (1967).
26. Kuroiwa, Y. *et al.* Manipulation of human minichromosomes to carry greater than megabase-sized chromosome inserts. *Nat. Biotechnol.* **18**, 1086–1090 (2000).
27. Brey, R.N. Molecular basis for improved anthrax vaccines. *Adv. Drug Deliv. Rev.* **57**, 1266–1292 (2005).
28. Raju, T.S., Briggs, B.J., Borge, M.S. & Jones, J.S.A. Species-specific variation in glycosylation of IgG: evidence for species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. *Glycobiology* **10**, 477–486 (2000).
29. Hering, D., Thompson, W., Hewetson, J., Little, S., Norris, S. & Pace-Templeton, J. Validation of the anthrax lethal toxin neutralization assay. *Biologicals* **32**, 17–27 (2004).
30. Pittman, P.R., Parker, & Friedlander, A.M. Anthrax vaccine: immunogenicity and safety of a dose-reduction, route-change comparison study in humans. *Vaccine* **20**, 1412–1420 (2002).
31. Semenova, V.A. *et al.* Mass value assignment of total and subclass immunoglobulin G in a human standard anthrax reference serum. *Clin. Diagn. Lab. Immunol.* **11**, 919–923 (2004).
32. Wakayama, T. *et al.* Cloning of mice to six generations. *Nature* **407**, 318–319 (2000).
33. Kubota, C., Tian, X.C. & Yang, X. Serial bull cloning by somatic cell nuclear transfer. *Nat. Biotechnol.* **22**, 693–694 (2004).
34. Mann, M.R. *et al.* Disruption of imprinted gene methylation and expression in cloned preimplantation stage mouse embryos. *Biol. Reprod.* **69**, 902–914 (2003).
35. Rideout, W.M., Eggan, K. & Jaenisch, R. Nuclear cloning and epigenetic reprogramming of the genome. *Science* **293**, 1093–1098 (2001).
36. Tamashiro, K.L. *et al.* Cloned mice have an obese phenotype not transmitted to their offspring. *Nat. Med.* **8**, 262–267 (2002).
37. Knight, K.L., Kingzette, M., Crane, M.A. & Zhai, S.K. Transchromosomally derived Ig heavy chains. *J. Immunol.* **155**, 684–691 (1995).
38. Nolan-Willard, M., Berton, M.T. & Tucker, P. Coexpression of mu and gamma 1 heavy chains can occur by a discontinuous transcription mechanism from the same unrearranged chromosome. *Proc. Natl. Acad. Sci. USA* **89**, 1234–1238 (1992).
39. Kamoda, S., Ishikawa, R. & Kakehi, K. Capillary electrophoresis with laser-induced fluorescence detection for detailed studies on *N*-linked oligosaccharide profile of therapeutic recombinant monoclonal antibodies. *J. Chromatogr. A.* **1133**, 332–339 (2006).