Characterization of immunoglobulin heavy chain knockout rats

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The rat is a species frequently used in immunological studies but, until now, there were no models with introduced gene-specific mutations. In a recent study, we described for the first time the generation of novel rat lines with targeted mutations using zinc-finger nucleases. In this study, we compare immune development in two Ig heavy-chain KO lines; one with truncated C\textsubscript{l} and a new line with removed JH segments. Rats homozygous for IgM mutation generate truncated C\textsubscript{l} mRNA with a de novo stop codon and no C\textsubscript{c} mRNA. JH-deletion rats showed undetectable mRNA for all H-chain transcripts. No serum IgM, IgG, IgA and IgE were detected in these rat lines. In both lines, lymphoid B-cell numbers were reduced \( >95\% \) versus WT animals. In rats homozygous for IgM mutation, no Ab-mediated hyperacute allograft rejection was encountered. Similarities in B-cell differentiation seen in Ig KO rats and ES cell-derived Ig KO mice are discussed. These Ig and B-cell-deficient rats obtained using zinc-finger nucleases-technology should be useful as biomedical research models and a powerful platform for transgenic animals expressing a human Ab repertoire.

Key words: B-cell deficient · Ig · Knockout rat · Transgenic rat · Zinc-finger nucleases

Introduction

The derivation of genetically engineered animals addresses basic biological problems, generates disease models and helps to develop new biotechnology tools [1, 2]. Although ES-cell-derived mice carrying introduced gene mutations have provided invaluable information, the availability of other species with engineered gene alterations is limited. For over 100 years, the rat has been an experimental species of choice in many biomedical research areas and in biotechnological applications [3, 4]. During the last 15 years, genetic engineering techniques have resulted in the generation of many transgenic and non-targeted mutated rats [1, 3, 4]. This has confirmed and complemented disease studies.

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but, as well as presenting biotechnological alternatives, also generated new paradigms. Nevertheless, the development of gene-targeted mutated rats was hampered by the absence of rat ES cells or robust cloning techniques. In 2008, rat ES cells were described [5, 6] but as yet there have been no reports on the generation of mutant rats from such cells. In 2009, we reported for the first time the generation of IgM-specific alterations directly in rats using zinc-finger nucleases (ZFN) [7–9].

ZFN are new versatile and efficient tools that have been used to generate several genetically modified organisms such as plants, Drosophila, zebra fish and rats as well as human ES cells [7]. ZFN are hybrid molecules composed of a designed polymeric zinc finger domain specific for a DNA target sequence and a FokI nuclease cleavage domain [10]. Since FokI requires dimerization to cut DNA, the binding of two heterodimers of designed ZFN-FokI hybrid molecules to two contiguous target sequences in each DNA strand separated by a 5–6 bp cleavage site results in FokI dimerization and subsequent DNA cleavage [10]. Following the DNA double strand break by a ZFN, the DNA can be repaired in two different ways; non-homologous end joining that generates short insertions or deletions at the cleavage site and therefore DNA frame shifts resulting in high incidence of functional KO or DNA double strand break by a ZFN, the DNA can be repaired in two different ways; non-homologous end joining that generates short insertions or deletions at the cleavage site and therefore DNA frame shifts resulting in high incidence of functional KO or homologous recombination using a DNA template which results in gene knockins that are either a perfect repair or, if a modified template is introduced, a sequence replacement [7].

Since Ig membrane expression on B lymphocytes is required for cell survival [11, 12], targeting IgM exons or the JH locus with ZFN was expected to generate non-homologous end joining mutations resulting in IgM-deficient rats and thus lacking mature B cells.

In this manuscript, we describe the phenotype of rats homozygous for a truncation in C1q and, separately, deletion of the JH locus. Both lines show no detectable Ig production and mature B-cell development. The availability of B-cell-deficient rats will permit to gain new insights of Ig function and development in health and disease. In addition, ZFN technology paves the way for simpler gene replacement and transgenic studies with the immediate aim of expressing human Ab repertoires in the rat.

Results

DNA and RNA analysis of the IgM mutation and of Ig transcription

Among several rat lines with IgM CH1 domain mutations [8], rat line 19 was bred to homozygocity. The mutation in this rat line comprised a 64 bp deletion in both alleles of the IgM CH1 domain gene (Fig. 1A, left) and no additional mutations in any of the ten genomic sequences most homologous to the one targeted [8]. Analysis of IgM mRNA by RT-PCR of JH1-Cq transcripts showed a shorter transcript in rats homozygous for IgM mutation (IgM KO rats) compared with WT (Fig. 1B, left). Analysis of IgG transcripts using RT-PCR of JH-Cγ showed the absence of mRNA in IgM KO rats and a strong signal of the expected size in WT rats (Fig. 1B, left). Heterozygous IgM KO rats showed the presence of IgM and IgG transcripts (data not shown). Digestion of the JH-Cq amplicon with DdeI resulted in the generation of a smaller band due to the 64 bp deletion (Fig. 1B, right). Sequencing of JH-Cq mRNA isolated from IgM KO rats showed a deletion of 64 bp and the generation of a stop codon (Fig. 1C).

Microinjection of rat zygotes with ZFN mRNA specific for the JH locus resulted in the generation of a mutant animal with a 2465 bp DNA deletion, spanning the entire locus (Supporting Information Data 1). In homozygous JH locus, mutant rats’ analysis of mRNA using primers spanning several VH or JH sequences to μCH2 (Fig. 1D) or Cγ sequences (data not shown) did not reveal detectable levels of transcripts. These results indicate that IgM KO rats have a deletion in the Cq1 domain that generated a stop codon, resulting in shorter IgM transcripts and no IgG transcripts. Rats homozygous for J deletion (JH KO rats) showed a large deletion and no detectable IgM or IgG transcripts.

Analysis of serum Ig

ELISA revealed undetectable levels for all Ig isotypes in IgM or JH KO rats analyzed (Fig. 2A). Heterozygous IgM KO animals and WT rats showed normal levels of IgM (1 246 ± 81 µg/mL), IgG (6 060 ± 1 356 µg/mL), IgA (65 ± 5 µg/mL) and IgE (2 845 ± 1 110 ng/mL). In mice, mutations in the IgM Cq1 exon have resulted in alternative splicing of the mutated region and shorter μ-chains were produced [13]. This suggested the possibility that shorter Cq polypeptides could be produced in mutant rats but failed to be recognized by the anti-μ mAb used for the ELISA. Although the absence of other Ig isotypes was not in agreement with this hypothesis, we aimed to formerly exclude the possibility by performing Western blot analysis using a polyclonal anti-μ Ab. Western blot analysis of different amounts of purified IgM showed that we could detect down to 7.8 ng/lane of μ-chains. WT sera diluted 1/100 gave a signal corresponding to 250 ng/lane (Fig. 2B, upper). Since 20 µL were loaded per lane, this corresponded to a detection limit of 390 ng/mL and 12.5 µg/mL μ-chains for purified and 1/100 diluted serum, respectively. Analysis of sera from three homozygous IgM (Fig. 2B, middle) or two JH (Fig. 2B, lower) KO rats showed undetectable levels of IgM (< 7.8 ng/lane) and thus below 12.5 µg/mL in serum. Sera from heterozygous IgM KO rats analyzed by Western blot showed normal size and concentration of μ-chains (data not shown).

These results indicated that both the IgM Cq1 and the JH mutation resulted in a complete absence of the production of all Ig isotypes.

Macro and microscopic analysis of lymphoid organs

The size of the spleens of IgM and JH KO rats was drastically reduced, whereas only some, but not all lymph nodes appeared to be slightly reduced. Thymus did not show obvious diminution (Fig. 3A). JH KO rats displayed an identical lymphoid organs macroscopic phenotype (data not shown).

Immunohistology showed that spleens of IgM KO rats were completely devoid of CD45RA+ B (Fig. 3B) and IgM+ B cells
As compared with WT animals, the TCRβ1 T-cell zones of IgM KO rats were well defined but reduced in size and a matching reduction was also seen for CD41 and CD81 T cells (Fig. 3B). Lymph nodes also showed a complete absence of CD45RA1B (Supporting Information Data 3) and of IgM1B cells (data not shown) but normal areas of TCR1, CD41 and CD81 cells (Supporting Information Data 3). Thymus also showed the absence of small clusters of CD45RA1B cells and normal areas of TCR1, CD41 and CD81 cells (Supporting Information Data 3).

These results indicate that B cells were virtually absent from secondary lymphoid organs in IgM and JH KO rats and as previously described for μMT KO and JH KO mice the number of T cells in spleen but not in lymph nodes or thymus was decreased [12, 14, 15].

Figure 1. Analysis of transcription products from ZFN IgM or J KO rats. DNA and mRNA were extracted from spleen cells from WT (n = 4), IgM KO (n = 4) and JH KO (n = 4) animals, amplified by PCR and RT-PCR, respectively, and sequenced. (A) WT DNA sequence of the IgM Cm1 exon aligned with the DNA sequence of homozygous of an IgM KO rat (line 19) showing a 64 bp deletion. ZFN sequences are underlined. (B) (Left) RT-PCR amplifications were carried out with oligo combinations JH1-Cm and JH-Cg using mRNA from leukocytes of WT and IgM KO homozygous rats. (Right) DdeI digests of Cm amplifications show a fragment of reduced size, 117 bp, as a result of the deletion (64 bp) in IgM KO homozygous rats. (C) In the Cm obtained mRNA sequence, primers are shown in italics, the DdeI site underlined, the stop codon in the mutated transcript in bold and the deleted sequence displayed above. (D) RT-PCR amplifications were carried out with oligo combinations of JH1-4 to μCH2 as well as different VH family primers (VH2, VH5/6/11, VH8 and VH1/7) to μCH2. GAPDH was used as positive control. Results from two representative animals (722 and 727) are shown.
Figure 2. Quantitative ELISA of serum Ig isotypes. Sera were collected from WT (n = 5), IgM homozygous KO (IgM KO, n = 6) and JH homozygous KO (J KO, n = 4) rats 12–18 wk old. (A) Isotype-specific and quantitative ELISA for the different rat isotypes. Each point represents the values of one animal and the horizontal bar the mean of each group of values. *p<0.01; **p<0.001 WT versus IgM KO or JH KO using Mann-Whitney test. (B) Western blot analysis of serum IgM. Purified rat IgM and sera harvested from WT, IgM homozygous KO and JH homozygous KO rats 12–18 wk old were analyzed by Western blot using polyclonal anti-rat μ heavy chain-specific Ab. (Upper) Purified rat IgM was diluted and the indicated total amount of Ab (ng) was loaded in each line. Serum from a WT rat was diluted as indicated and 20 μL was loaded by line. (Medium) Serum from a WT rat and sera from three IgM KO rats were diluted as indicated. (Bottom) Serum from a WT rat and sera from two JH KO rats were diluted as indicated.

Figure 3. Macroscopic and immunohistology analysis of lymphoid organs. Lymphoid organs were harvested from WT (n = 4) and IgM homozygous KO (IgM KO, n = 6) rats 12–18 wk old. (A) Macroscopic analysis of spleen, lymph nodes and thymus. (B) Immunoperoxidase staining of spleen cryostat sections using mAb directed to the defined cell markers and cell types between parentheses and counterstained with Mayer’s hematoxylin.
Flow cytometry analysis for quantification of immune cells in bone marrow (BM) and lymphoid organs

To better define the blockade in B-cell differentiation and to quantify the absolute numbers of different cell subsets, we evaluated the single-cell composition in the various lymphoid organs. Using CD45R (B220) and IgM as markers, several B-cell populations could be identified in the rat [16]: pro–pre B (IgM<sup>−</sup> CD45R<sup>low</sup>), immature (IgM<sup>low</sup> CD45R<sup>low</sup>), transitional (IgM<sup>high</sup> CD45R<sup>low</sup>), marginal zone (IgM<sup>high</sup> CD45R<sup>high</sup>) and mature (IgM<sup>low</sup> and high CD45R<sup>high</sup>). The analysis of IgD allowed a further subdivision of IgM<sup>+</sup> B cells as IgD<sup>low</sup>/CD45R<sup>−</sup> marginal zone and IgD<sup>+</sup>/follicular B cells and IgM<sup>low</sup>/IgD<sup>−</sup> as immature/transitional B cells [17]. Analysis of WT rats showed mostly pro–pre B and some immature B cells in BM, mostly immature and some mature in lymph nodes and similar proportion of immature and transitional cells (Fig. 4A and Table 1). Spleen and lymph nodes of IgM or JH KO rats showed barely detectable IgM or IgD positive cells (Fig. 4A, Table 1 and Supporting Information Data 4). The total number of cells in the spleen and lymph nodes of IgM or JH KO rats were drastically decreased versus WT rats (Table 1). IgM<sup>+</sup> and CD45R<sup>−</sup> cells in the spleen of IgM or JH KO rats were drastically decreased versus WT rats (IgM<sup>−</sup>: 0.7 and 2.28%, respectively; CD45R<sup>−</sup>: 1.6 and 4.3%, respectively) (Table 1). FACS analysis showed the presence of a small population of CD45R<sup>+</sup>IgM<sup>−</sup> cells in spleen (Fig. 4A, Table 1). Immunohistology revealed their location mainly in the spleen red pulps (data not shown). Using several markers, we confirmed that the phenotype of CD45R<sup>−</sup> cells in IgM KO rats corresponded to the previously described phenotype of rat pDC [18] (data not shown).

In lymph nodes, absolute numbers of IgM<sup>+</sup> or CD45R<sup>−</sup> cells were greatly reduced in IgM or JH KO rats versus WT controls (~4 and ~4.5%, respectively) (Table 1).

In BM of IgM or JH KO rats, we observed no immature or mature B cells and greatly reduced proportion of pro–pre B cells (IgM<sup>−</sup> CD45R<sup>low</sup>) (Fig. 4A). The absolute number of mononuclear cells was significantly reduced in IgM and JH KO versus WT rats (42.2 and 56.7%, respectively) (Table 1) and numbers of pro–pre B cells (IgM<sup>−</sup> CD45R<sup>low</sup>) in IgM, JH KO and WT were 12.8 and 22.4%, respectively, versus WT (Table 1).

T cells in spleen, as defined by double staining using anti-TCRαβ and anti-CD4 or anti-CD8 Ab, showed an increased proportion of TCRαβ<sup>−</sup> cells compared with WT rats (~85% in IgM and JH KO rats versus ~40% in WT animals), both of the CD4<sup>+</sup> and CD8<sup>+</sup> subtypes (Fig. 4B). Despite this increase, the total numbers of spleen cells in IgM and JH KO rats were only 13.6 and 16.6%, respectively, compared with WT spleen cells and thus the total numbers of TCRαβ<sup>−</sup> cells in IgM and JH KO rats were 30 and 33.7%, respectively, versus WT (*p < 0.05 for both IgM or JH KO versus WT) (Table 1).

Despite the fact that cell numbers in the lymph nodes were considerably decreased in IgM or JH KO versus WT rats (43 and

Figure 4. Analysis of lymphoid cell subsets by cytofluorimetry. Lymphoid organs were harvested from WT (n = 4), IgM homozygous KO (IgM KO, n = 6) and JH homozygous KO (JH KO, n = 4) rats 12–18 wk old. (A) Cell suspensions from spleen, lymph nodes and BM were labelled with anti-IgM (abscissa) and anti-B-cell-specific CD45R mAb (ordinates) and analyzed by flow cytometry. Gating strategy is shown in Supporting Information Data 2 and the same one was used for all FACS analysis. The square windows represent sequential stages of B-cell development (pro–pre B (Pro B IgM<sup>−</sup> CD45R<sup>low</sup>), immature (imm. IgM<sup>low</sup> CD45R<sup>low</sup>), transitional (Trans. IgM<sup>high</sup> CD45R<sup>low</sup>) and mature (mat IgM<sup>low</sup> and high CD45R<sup>high</sup>)) and the figures the percentages of cells in each stage. Representative results from four to six animals of each group. (B) Flow cytometry analysis of cell suspensions from spleen labelled with anti-TCRαβ (abscissa) and anti-CD4 or anti-CD8 mAb (ordinates).
CD4 cells (ab 39%, respectively), T cells were not significantly reduced (Table 1) due to a significantly increased proportion of TCRμT cells ( ~95% for both KO versus ~78%, respectively) with the CD4+ or CD8+ surface marker (Supporting Information Data 2).

In BM, the proportion of TCR+ cells was increased in IgM or JH KO versus WT rats (both ~35 versus ~10%, respectively) in both compartments, TCR+CD4+ and TCR+CD8+ (Supporting Information Data 2). The total number of T cells was also significantly increased in IgM or JH KO versus WT (275 and 201%, respectively) (Table 1).

In thymus of IgM or JKO rats, the proportion of TCR+, TCR+CD4+ and TCR+CD8+ cells (Supporting Information Data 3) as well as the total number of T cells (Table 1) were comparable.

IgM KO rats, 1 year old showed the same cellular phenotype and the absence of Ig as younger animals (data not shown).

Thus, both IgM and JH KO rats showed a blockade on B-cell differentiation in the earliest stages of B-cell development in BM with greatly reduced B cells in peripheral lymphoid organs. Total T CD4+ and T CD8+ cells were also significantly decreased in spleen but not in lymph nodes. T cells were increased in BM and maintained in the thymus of IgM or J KO versus WT rats.

### Heart allograft survival

To test in vivo for the absence of B cells, we used a model of hyperacute heart allograft rejection in which increased anti-donor Ab are the first rejection mechanism. In this model, recipients were immunized against donor antigens by multiple skin transplants from MHC-mismatched donor prior to heart transplantation from the same donor. WT recipients without previous donor immunization rejected donor hearts in 7 days (p < 0.05). Immunized recipients exhibited accelerated rejection in hours (1 h40, 5 h00 and < 8 h00) with high titers of anti-donor Ab (Fig. 5A and B). On the contrary, IgM KO rats showed significantly prolonged survival of transplanted hearts (144 h (d6), 168 h (d7), 456 h (d19), 480 (d20); p < 0.05 versus WT) (Fig. 5A). Importantly, flow cytometric analysis showed that IgM KO rats did not produce Ab binding to donor cells (Fig. 5B).

Thus, B-cell and Ab-deficient animals showed delayed allograft rejection after repeated anti-donor stimulation in a model of Ab-mediated rejection.

### Discussion

Although the rat has been a major experimental species in physiological studies for many years, the lack of robust genetic engineering technologies to generate gene-specific mutations hampered its use in many other models [1, 3, 4, 7]. The cloning of the rat through nuclear transfer has been described several years ago [19] but a source of suitable cells in which gene targeting and selection of mutants is feasible without losing cloning potency is lacking. Analogously, rat ES cells [5, 6] and induced pluripotent stem cells [20] have been recently
controls n=3

IgM KO n=4

IgM KO n=4

controls n=3

Percent survival

Time in hours

Days

IgM

Mean fluorescence

IgG

Mean fluorescence

B

Figure 5. Hyperacute allograft rejection mediated by alloAb. Recipient WT or IgM KO rats (both MHC RT-1a haplotype) were grafted three times at 1-wk intervals with skin from LEW.1A donors (MHC RT-1a haplotype) and 1 wk later grafted with a LEW.1A donor heart. (A) Kaplan–Meier graft survival curve showing graft rejection in some hours for WT rats and 1 wk later grafted with a LEW.1A donor heart. (A) Kaplan–Meier analysis of IgM (upper panel) and IgG (lower panel) anti-LEW.1A alloAb levels in sera diluted 1/10 from day 0 before skin transplantation up to the moment of heart rejection (for WT and one IgM KO) or up to day 43 in non-rejecting IgM KO. Results are shown in mean channel fluorescence after subtraction of mean channel fluorescence obtained incubating sera with recipient T cells ± SD. *p<0.05 IgM KO versus WT for all time points using Mann–Whitney test.

IgM KO n=4

controls n=3

described and may eventually allow generation of precise gene modifications as obtained in mice. However, currently, there are no reports of gene KO rats from such cells. KO rats have been described using chemical mutagens [21] or transposons [22] but these techniques, although very useful, generate random non-controlled mutations and are thus labour intensive and expensive. The first gene-specific KO rats with mutations in IgM (phenotyped here) and Rab38 endogenous loci as well as a transgenic GFP were generated using ZFN [7–9].

ZFN provide several advantages to generate novel rat lines carrying mutations in specific genes. The most important ones are the capacity to target specifically a given gene and the high efficiency of the procedure. As far as specificity is concerned, we showed that the most homologous non-related sequences in the rat genome to the one targeted by the IgM ZFN did not show non-specific mutations [8, 9]. Although it needs to be established whether any given gene can be targeted by ZFN, our experience with other ZFN, for example directed against rat λ and κ light-chain genes (our unpublished results) as well as the IL-2 receptor γ locus gene [23], have all resulted in successful KO. The other major advantage of ZFN is the speed of the procedure since KO rats can be generated in about 4 months in both inbred and outbred strains [8, 9, 23]. Finally, mutations are definitive and transmitted to the progeny.

Our characterization of IgM KO and JH KO rats confirm the previous findings in μMT or J KO mice [11, 12] and immuno-deficient human patients [24, 25] that the absence of membrane Ig expression results in the absence of B cells. On the contrary, IgM deletion [26] or truncation [13] in mice permitted expression of other heavy chains and allowed B-cell development and maturation due to replacement of IgM by IgD. Similarly to humans [24, 27], IgM KO rats showed only 5% of normal levels of BM pro-pre B cells, whereas μMT mice showed normal levels of BM pro-pre B cells [11]. In this regard, deletion in mice of the Ig JH region resulted in a block of Ig gene expression and B-cell development at the pro-B-cell stage [12] as for JH KO rats. Thus, like μMT mice in which transcription and translation of μ-chain occurred but did not result in expression of membrane-bound IgM and like JH KO mice, IgM KO rats showed a shortened μ transcript and the absence of Ig polypeptide production and therefore a very early B-cell block. As for mice and human cells, an enigma still persists on how B-cell levels can be suppressed early or potentially, after rearrangement at the pre-BCR stage but before a fully functional μ polypeptide is expressed. An answer to this may be dependent on the level of early control of the IgH locus when chromatin is opening and antisense transcription will be initiated before D to J recombination [28]. It is possible that strain-specific parameters as well as size and position of the removed or targeted region may determine the B-cell block.

Another difference with IgM KO mice [11] is that these mice showed normal levels of IgA and absence of all other Ig isotypes [29], whereas IgM KO rats showed complete deficiency of all isotypes including IgA. Analogously to IgM KO rats, patients with deletion of the μ locus also result in the absence of Ig production for all isotypes including IgA [25].

Since in contrast to mice, only 1% of cells recovered from the peritoneal cavity of rats are B cells [17], we did not analyze this compartment.

In IgM or JH KO rats’ T-cell numbers in spleen but not in lymph nodes were decreased, as described for μMT mice [14, 15]. In μMT mice, this was due to the lack of production of lymphotixin α1β2 by B cells, required for CCL21 and stromal cell development, and as yet to be defined mechanism(s) for the promotion of T-cell numbers [14]. This decrease in spleen T cells in μMT mice did not result in impairment of several immune responses, but depletion of CD8+ cells did impair heart allograft rejection.
by cellular immune responses when initiated in the spleen but not in lymph nodes [15]. We also observed that T cells were significantly increased in the BM of IgM KO rats and this vascular compartment of T cells could replace at least in part the reduced pool of spleen T cells for immune responses mainly taking place in the blood and spleen. Therefore, care should be taken when analyzing T-cell responses in B-cell-deficient animals, in particular when immune responses are mediated in the vascular compartment and spleen as compared with other tissues. Further experiments are needed to analyze this point in IgM or JH KO rats. As far as Ab-mediated hyperacute allograft rejection is concerned, IgM KO rats showed a significantly delayed rejection which was associated with undetectable levels of alloAb, as previously described in μMT mice [30].

In conclusion, we generated a new rat KO line by ZFN-targeted deletion of the J locus and we describe that both IgM KO rats and JH KO rats are B cell and Ig deficient. These animals will be useful models to explore the role of B cells and Ab in different pathophysiological processes as organ rejection. They will also be useful for the generation of rats expressing a human Ab repertoire, an important application of transgenic animals [2].

Materials and methods

Generation of IgM or J KO rats

Sprague–Dawley WT, IgM KO and JH KO rats analyzed were 10–18 wk old. In addition, IgM KO over 1 year old were compared with younger animals. Animals were bred at Charles River under specific pathogen-free conditions. The generation of heterozygous IgM KO rats using ZFN has been described previously [8, 9]. JH KO rats, generated using ZFN (Sigma) targeting sequences upstream and downstream of the rat JH locus (Supporting Information Data 1) (ZFN1: CAGGATGTGCCCATCCAGCTGAGTTAAGGTGGAG; ZFN2: CAGGACAGGACACCTGCAGAGTGGCAGAAAGGT; bindings sites underlined) were designed and validated biochemically in vitro as described previously [31]. Pronuclear injections of in vitro-transcribed mRNA-encoding ZFN were performed as described previously [8, 9] using Sprague–Dawley rats. Offspring with large deletions was identified by PCR using the primers GATTTACTGAGG; GAPDH F: CAGTGCCAGCTCGTCTCAT; GAPDH R: GAGTCATGGTCA; JH3: TACTGGGGCCACAGGT; JH4: TGCCTGGGGTGCAAGGAGCTTCAGTCA; VH2: CAGGTGCAGCTGAGAAGWGCAG; VH8: CAGGTATCCTGAAGAGTCTGG; VH1_7: CAGGTC-CAGCTGCWGSARTCTG; μCH2R GCTTCTAGATGTT-CAGITGGCTATATGC; γCH2: GTTTGGAGATGCTTTCTGC-ATGGG; GAPDH F: CAGTGCCAGCTGCTCCTCAT; GAPDH R: AGGGGCACTCCAGTCTTTC. GoTaq® Green Master mix (Promega) was used as per the manufacturer’s instructions (www.promega.com) with amounts of sample cDNA adjusted by comparing GAPDH band strength. Annealing temperatures used for the PCR were set at the lowest primer Tm – 5°C (http://www.sigma-genosys.com/calc/DNACalc.asp). The reaction conditions were 95°C for 2 min, 34 cycles of 95°C for 20 s and 70°C for 40 s, followed by 70°C for 5 min RT-PCR products were cleaned up using SureClean (Bioline) digested with Ddel (NEB) or sequenced directly.

Identification of cell subsets by flow cytometry

Cell suspensions were washed and adjusted to 5 × 10⁵ cells/well in PBS-1% BSA-0.1% Azide. The different B-cell subsets were identified using mouse anti-rat IgM FITC-labelled mAb (MARM 4, Jackson Immunoresearch Laboratories) in combination with anti-CD19 mAb (G4.18 and R7.3, Serotec). The incubation period was 30 min at 4°C and for the analysis an FACS CantoII flow cytometer and FlowJo software (Becton Dickinson, Pont de Claix, France) were used. T cells were detected using anti-CD3 and anti-αβTCR mAb (G4.18 and R7.3, both from BD biosciences) as described previously [32].

Identification of cell subsets by immunohistochemistry

Tissue biopsies were prepared as described previously [32]. BM cells were obtained by flushing one femur with PBS. Cell suspensions were then pelleted and red blood cells were removed by erythrocyte lysis. Cell suspensions were washed twice and passed through a nylon gauze before counting the cells using an haemocytometer. Part of the cell suspensions was used for mRNA and DNA extraction and others for flow cytometry analysis.

Analysis of transcription products from KO rats

RNA was extracted from rat spleen cells using TRizol (Invitrogen), stored in RNAlater (Ambion) and reverse transcribed at 42°C with BioScript (Bioline, London, UK). PCR reactions were set up using rat JH or VH forward primers with μCH2 or γCH2 reverse primers. Sequences of primers from ‘s’ to ‘t’ were as follows: JH1: TCTCGGGGCCCAGGAACATGCTCA; JH2: TACTGGGGCCAGAGTGCTATGCTCA; JH3: TACTGGGGGCAAGGCCCATCTGCTCA; JH4: TGCTCGGGGTCAAGGAGCTTCTAGTCA; VH1: CAGGTGCAGCTGAGAGWGCACG; VH2: CAGGTATCCTGAAGAGTCTGG; VH1_7: CAGGTC-CAGCTGCWGSARTCTG; μCH2R GCTTCTAGATGTT-CAGITGGCTATATGC; γCH2: GTTTGGAGATGCTTTCTGC-ATGGG; GAPDH F: CAGTGCCAGCTGCTCCTCAT; GAPDH R: AGGGGCACTCCAGTCTTTC. GoTaq® Green Master mix (Promega) was used as per the manufacturer’s instructions (www.promega.com) with amounts of sample cDNA adjusted by comparing GAPDH band strength. Annealing temperatures used for the PCR were set at the lowest primer Tm – 5°C (http://www.sigma-genosys.com/calc/DNACalc.asp). The reaction conditions were 95°C for 2 min, 34 cycles of 95°C for 20 s and 70°C for 40 s, followed by 70°C for 5 min RT-PCR products were cleaned up using SureClean (Bioline) digested with Ddel (NEB) or sequenced directly.

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(5 μm) from tissues were thawed, fixed in acetone (10 min at room temperature) and incubated with mAb (1 h at room temperature, 10 μg/mL) recognizing CD45RA (OX33), zυTCR, CD8 (OX8) and CD4 (W3.25), followed by biotin-conjugated anti-mouse Ab (Jackson ImmunoResearch Laboratories) as described previously [31]. Ab binding was detected by incubation with HRP-conjugated streptavidin using Vector® VIP (Vector Laboratories, Burlingame, CA, USA) as a substrate. Tissue sections were counterstained with Mayer’s hematoxylin and lithium carbonate.

Detection of Ig in rat sera by quantitative ELISA

Serum Ig concentrations were determined by a quantitative ELISA, using plates coated with isotype-specific mouse mAb anti-rat Ab to IgM (MARM-4), IgG (MARG), IgE (MARE) or IgA (MARA) (all from Abd Serotec, Jackson ImmunoResearch, BD Biosciences) at 5 μg/mL in PBS overnight at 4°C. After washing with PBS-Tween 0.5%, plates were blocked for 2 h at room temperature with PBS-BSA 3% w/v. Sera were diluted in PBS starting at 1/10 and incubated for 2 h at 37°C. After washing, a mixture of mouse mAb anti-rat κ and anti-λ chain-specific peroxydase-conjugated Ab (clone MARK-1/MARL-15, Abd Serotec) were added at a dilution of 1:2000 in PBS for 1 h. Purified rat Ab IgM, IgG, IgA and IgE isotypes (Abd Serotec, Jackson ImmunoResearch, BD Biosciences) were added at different known concentrations and used as standard. After three washes, TMB substrate reagent (Becton Dickinson) was added and the reaction was stopped after 10 min by the addition of 2 N H2SO4. Absorbance was read at 490 nm. Serum Ig concentrations were determined by extrapolating absorbance values of sera dilutions in the linear range of the dilution curves to those of isotype standard controls.

Western blot analysis

Protein concentration of serum was measured (BCA Protein Assay Kit, Pierce, Rockford, IL, USA). A standard curve dilution of monoclonal purified rat IgM and dilutions of rat serum (1/10 and 1/100) (20 μL/line) were electrophoresed in 12% SDS-polyacrylamide gels. After semi-dry transfer, the nitrocellulose membranes were blocked in 5% dry milk in PBS with 0.05% Tween-20 for 1–2 h and incubated overnight at 4°C with Peroxydase-conjugated goat anti-rat IgM μ-specific Ab (from Jackson Laboratories) at 1 μg/mL. The binding was visualized with enhanced chemiluminescence and quantified using a Fuji LAS 4000 (Fujifilm) imaging system and Multi Gauge V3.0 software (Fujifilm).

Heart allograft transplantation and anti-donor Ab

IgM KO rats (haplotype RT1u for MHC I and II) were immunized against donor LEW.1A alloantigens (haplotype RT1u for MHC I and II) by three successive skin grafts separated by 1 wk each and grafted with a LEW.1A heart 1 wk after the last skin transplant. Heart transplantation was performed heterotopically in the abdomen. Heart allograft survival was evaluated through abdominal palpation and rejection was defined as total cessation of beating and confirmed by visual inspection after laparotomy [32, 33]. Anti-donor Ab were analyzed in the sera of WT or IgM KO rats harvested at day 0 before skin transplantation, at days 20 and 30 after skin transplantation and at rejection or later time points if hearts were not rejected. Heat-inactivated sera (dilutions 1/10, 1/100 and 1/1000 and 1/5000) were incubated with donor T cells, washed and alloAb bound were detected using rat anti-IgG or IgM-specific Ab. As negative control, sera were incubated with recipient T cells. Results were reported as the mean channel fluorescence using donor T cells – mean channel fluorescence using donor T cells ± SD.

Statistical analysis

Results are presented as the means ± SD. Statistical analyses were performed by a Mann–Whitney test for laboratory analyses and Kaplan–Meier log rank test for graft survival using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, USA). Differences associated with probability values of p<0.05 were considered statistically significant.

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