Gain of function of the immune system caused by a ryanodine receptor 1 mutation

Mirko Vukcevic¹, Francesco Zorzato¹², Simone Keck³, Dimitrios A. Tsakiris⁴, Jennifer Keiser⁵, Rick M. Maizels⁶ and Susan Treves¹²

¹Departments of Anaesthesia and Biomedicine, Basel University Hospital, Hebelstrasse 20, 4031 Basel, Switzerland; ²Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy; ³Laboratory of Transplantation Immunology, Departments of Biomedicine and Nephrology, University Hospital Basel and University of Basel, Hebelstrasse 20, CH-4031 Basel; ⁴Department of Haematology, University Hospital Basel, CH-4031 Basel, Switzerland; ⁵Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, PO Box, CH-4002 Basel, Switzerland; ⁶Institute of Immunology and Infection Research, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, U.K.

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To whom correspondence should be addressed:
Susan Treves,
Departments of Anaesthesia and Research, Basel University Hospital,
Hebelstrasse 20, 4031 Basel, Switzerland.
Tel: +41612652373; Fax: +41612653702;
E-mail: susan.treves@unibas.ch

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SUMMARY

Mutations in RYR1, the gene encoding ryanodine receptor 1, are linked to a variety of neuromuscular disorders including Malignant Hyperthermia (MH) a pharmacogenetic hypermetabolic disease caused by dysregulation of calcium in skeletal muscle. RYR1 encodes a calcium channel that is predominantly expressed in skeletal muscle sarcoplasmic reticulum where it is involved in releasing the calcium necessary for muscle contraction. Other tissues however, including cells of the immune system, have been shown to express ryanodine receptor 1; in dendritic cells its activation leads to increased surface expression of major histocompatibility complex II molecules and provides synergistic signals leading to cell maturation. In the present study we investigated the impact of an MH mutation on the immune system by studying the RYR1Y522S knock in mouse. Our results show that there are subtle but significant differences both in resting non-challenged mice as well as in mice treated with antigenic stimuli, in particular the knock-in mice (i) have dendritic cells that are more efficient at stimulating T cell proliferation; (ii) have higher levels of natural IgG1 and IgE antibodies and (iii) are faster and more efficient at mounting a specific immune response in the early phases of immunization. We suggest that some gain of function MH-linked RYR1 mutations may offer selective immune advantages to their carriers. Furthermore, our results raise the intriguing possibility that pharmacological activation of RyR1 may be exploited for the development of new classes of vaccines and adjuvants.
INTRODUCTION

The ryanodine receptor (RyR1) intracellular Ca\textsuperscript{2+} channel is preferentially expressed in skeletal muscle where it plays a central role in excitation-contraction coupling by releasing calcium from the sarcoplasmic reticulum. Recently it was shown that RyR1s are also expressed in other cell types, including neurons, smooth muscle cells and immune cells, specifically in B-lymphocytes and dendritic cells (DC) (Sei et al., 1999; Girard et al., 2001; Bracci et al., 2007; Uemura et al., 2007; O'Connell et al., 2002). DCs are the most potent antigen presenting cells connecting innate and adaptive immunity. They are located in peripheral tissues where they continuously sample the environment for the presence of foreign antigens; when these are encountered DCs process them and then migrate to secondary lymphoid organs where they present the processed antigens in conjunction with Major Histocompatibility Complex II (MHCII) molecules to T-lymphocytes and initiate a specific immune response ((Banchereau et al., 2000). Studies on the role of RyR1 in immune cells in vitro has established that in B-lymphocytes its activation is coupled to cytokine release (Girard et al., 2001) whereas in DCs it leads to enhanced maturation, release of pro-inflammatory cytokines and enhanced ability to prime T-cell (Bracci et al., 2007).

In humans, mutations in RYR1 are associated with several neuromuscular disorders, including Malignant Hyperthermia, Central Core disease, some forms of Multiminicore disease, Centronuclear myopathy and congenital fibre type disproportion. More than 200 causative mutations have been identified in patients and though they have not all been characterized functionally, Malignant Hyperthermia (MH) causative mutations are characterized by “gain of function“, whereby they increase the sensitivity of the RyR1 Ca\textsuperscript{2+} channel to activation (Treves et al., 2008; Robinson et al., 2006). Indeed MH Susceptibility (MHS) is characterized by abnormal release of Ca\textsuperscript{2+} from the sarcoplasmic reticulum, metabolic acidosis, increase in body temperature and rhabdomyolysis after contact with a trigger agent. To date the functional effects of RYR1 mutations have been extensively studied in muscle cells and more recently, in the central nervous system (De Crescenzo et al., 2012) but no data is available on if and how mutations in RYR1 affect the immune system. In the present study we analysed the general characteristics of the
immune system of a mouse model knocked in for the RYR1$_{Y522S}$ mutation, a mutation that in humans has been shown to be causative of MH. Indeed mice carrying the mutation at the heterozygous state (HET RYR1$_{Y522S}$) are MHS, heat intolerant and develop an MH reaction when exposed to anaesthetics, whereas at the homozygous state the mutation causes death soon after birth possibly due to breathing impairment (Chelu et al., 2006). Our results show that there are subtle differences in the immune system of the heterozygous RyR1$_{Y522S}$ knock in mice compared to their wild type littermates, even in non immunized animals; specifically their DCs have a more mature phenotype, are more potent at stimulating T-cells and the serum concentrations of circulating natural IgG$_1$ and IgE are significantly increased. Moreover, following a primary antigenic challenge, heterozygous RYR1$_{Y522S}$ mice produce higher levels of antigen-specific IgG. These results support the intriguing possibility that some RYRI mutations exert beneficial effects on the immune system.
RESULTS

Phenotypic and functional characteristic of dendritic cells from the HET RYR1_{Y522S} knock in mouse:

It has previously been shown that human monocyte-derived DCs and mouse bone marrow-derived DC express RyR1 (Bracci et al., 2007; O'Connell et al., 2002). In this study we isolated CD11c+ cells from mouse spleens and confirm the presence of the RyR1 transcript. As shown in figure 1A, RYR1 transcripts in DCs from wild type (WT) and heterozygous (HET) RYR1_{Y522S} knock in mice differ, as the presence of the T>C substitution results in the appearance of a BplI restriction site in the HET RYR1_{Y522S} mice (Chelu et al., 2006). The presence of the RYR1 MH causing mutation in DCs caused a small but significant increase in the resting [Ca^{2+}]_{i} (fig.1B) as well as a significant increase in the surface expression of the maturation marker CD83 (fig. 1C). An increase in CD83 surface expression could be induced in DCs from WT mice by stimulation with 10 mM caffeine (inset Fig 1C), indicating that DCs are endowed with a pool of CD83 molecules that can be expressed on the plasma membrane by RyR1 activation. Figure 1D shows results obtained by real time PCR of common DC maturation markers; the relative expression of CD83, CD86, IL-12 and IL-23 do not differ between HET RYR1_{Y522S} and WT littersmates, indicating that the presence of the mutation does not affect transcription of these genes but rather affects the Ca^{2+} dependent release of CD83 onto the plasma membrane.

We next investigated if the increase in CD83 surface expression in DCs from the HET RYR1_{Y522S} mice was paralleled by functional changes, as monitored by the mixed lymphocyte reaction. DCs were isolated from spleens of either WT or HET RYR1_{Y522S} littermates (both having the C57BL/6 background) and incubated with different amounts of T-cells from Balb/c mice. The capacity of DCs to stimulate T-cell proliferation was assayed by measuring [^{3}H]thymidine incorporation. As shown in fig 2A starting from a DC:T-cell ratio of 1:20, cells isolated from HET RYR1_{Y522S} (grey bars) mice were significantly more efficient at stimulating alloreactive T cell proliferation compared to cells from their WT littersmates. Furthermore, supernatants collected 72h after co-culture of T cells and DCs from the HET RYR1_{Y522S} mice contained significantly higher levels of IFN-γ, with no difference in the amount of TNF-α, and IL-4 (fig. 2B). No IL-10 was
detected in the supernatants of the mixed lymphocyte reactions from either group (not shown).

**Blood counts, immune cell subpopulations and natural Ig in non-challenged mice:**

The total number of circulating leukocytes, erythrocytes and reticulocytes were similar in WT and HET RYR1Y522S mice (supplementary Table 1). There was a small but significant increase in the total number of circulating lymphocytes but close evaluation did not reveal any differences in the % CD4 or CD8 positive cells (not shown). Splenocytes isolated from HET RYR1Y522S and WT littermates were labelled with anti-CD4, CD8, CD3, CD19, MHCII, CD11c, CD11b, CD83 and the % positive cells was monitored by FACS. Supplementary figure 1 shows that there were no significant difference in the % of DCs, B-lymphocytes and macrophages; there was a small but significant increase in CD3+ve T-cells (mean % ± s.e.m. was 29.4±0.78% vs 33.3±0.9% in 12 WT and 10 HET RYR1Y522S littermates, respectively P<0.003). A closer evaluation revealed that this is due to an increase in the % CD4+ve subpopulation (mean % ± s.e.m. were 54.8± 0.4% and 58.7± 0.5% P< 0.0001 in WT and HET RYR1Y522S littermates, respectively). Spleen weights were similar in WT and HET RYR1Y522S littermates (mean ± s.e.m. weights were 93.1±4.8 and 100.6±4.8 mg, respectively).

When examining the levels of circulating immunoglobulins in non-immunized mice, we noticed that HET RYR1Y522S mice have significantly higher levels of natural IgG1 and IgE compared to their WT littermates but show no significant differences in the levels of IgM, IgG2a, IgG2b or IgG3 (figure 3). Such changes were not accompanied by changes in IL-4, IL-10 or IL-6 as the circulating levels of these cytokines remained undetectable in both mice groups. Surprisingly there were no changes in the population of splenic Th2 cells, as WT and HET RYR1Y522S mice showed similar levels of expression of the Th2-specific surface markers T1/ST2, OX40 and inducible T-cell co-stimulator (COS) (Clay et al., 2009; Withers et al., 2009). Furthermore no changes were observed in the levels of the Th1 and Th2 commitment transcription factors T-box expressed in T-cells (Tbet) and GATA-3 (Zhu et al., 2010) (supplementary Table 2). In addition, no differences were found in the native spleen B-cell populations as determined by the expression of surface immunoglobulins (IgM, IgG1, IgE), nor in the spleen populations of plasma cells, as determined by the expression of CD138 (supplementary Table 2). Re-
stimulation of splenic T-cells in vitro with CD3/CD28 beads did not reveal any difference
between HET RYR1Y522S and WT T-helper cell commitment since intracellular cytokine
staining related to Th1/Th2/Th17 profiles were not changed (supplementary Table 2).

**Enhanced humoral immune response in HET RYR1Y522S knock in mice after
antigenic challenge:** Since HET RYR1Y522S knock in mice express high levels of natural
circulating immunoglobulins we investigated if they showed an enhanced immune
response after an antigenic challenge. To this end we followed the immune response to (i)
ovalbumin (OVA), a well-characterized antigen for studying murine immune functions,
and (ii) *Heligmosomoides polygyrus (H.p.) bakeri*, a helminthic parasite. In the first case,
mice were immunized intraperitoneally with different amounts of ovalbumin (10, 20, 50,
100 and 200 µg) and the serum levels of OVA-specific immunoglobulins were
determined after 7, 14 and 21 days. Figure 4A shows that after 7 days HET RYR1Y522S
mice immunized with 100 µg OVA produced double the amount of OVA-specific IgG1
than their WT littermates. With higher or lower concentrations of OVA IgG1 levels were
similar in WT and HET RYR1Y522S littermates (not shown). No significant differences
were found in OVA-specific IgG1 levels 14 and 21 days after the primary challenge
(figure 4A).

Because HET RYR Y522S mice have elevated natural IgE and because immune
responses to helminthic infections preferentially elicit IgE responses, we challenged HET
RYR1Y522S knock in and control WT littermates to infection with *H.p. bakeri*. After 7 and
14 days the serum was checked for parasite-specific antibodies and at 15 days the mice
were sacrificed, the worm titre per mouse and white blood cell counts evaluated. No
differences in the number or type of white blood cells were observed between WT and
knock in mice (supplementary Table 3). Antibody titres to two worm-specific antigens
were assessed by enzyme linked immunosorbent assay (ELISA) by monitoring reactivity
against: (i) soluble extracts of whole worms and (ii) *H. p. bakeri* excretory-secretory
(HES) Ag, a major target of the murine primary immune response. Figure 4B and
supplementary Tables 4 and 5 show that after 7 days there is a significant increase in
parasite-specific IgG1, both to whole worm extracts and to the HES Ag, in HET
RYR Y522S knock in mice compared to WT littermates. At 14 days parasite-specific IgG1
reach the same levels in WT and knock in mice. No correlation was found between the
titres of worm/HES-specific antibodies and worm load.
DISCUSSION

A large number of studies have investigated the effects of RYR1 mutations on muscle cell calcium homeostasis but very little information is available concerning how such mutations affect other RyR1 expressing cells including immune cells. In this study we explored the consequences of the MHS-causative RYR1 Y522S mutation by analysing the phenotypic and functional characteristics of DCs from HET RYR1Y522S mice as well as their immune response to antigenic challenges.

Effect of the RYR1 Y522S mutation on the unchallenged immune system

Splenic CD11c+ DCs from the HET RYR1Y522S mice exhibit elevated resting calcium levels, a result which is consistent with the finding that most dominant RYR1 mutations linked to MHS cause an increase in the resting [Ca\(^{2+}\)] concentration in skeletal muscle cells and B-lymphocytes (Ducreux et al. 2004; Lopez et al., 2005; Levano et al., 2009; Vukcevic et al., 2010). Thus we hypothesized that the HET RYR1Y522S knock in mouse, which expresses a “gain of function” mutation (Chelu et al., 2006) would be a useful tool to study in depth the role(s) of RyR1-dependent signalling in the immune system. We first focused our efforts on identifying differences in the “resting“ characteristics of the immune system by analysing the phenotype of freshly isolated unchallenged DCs and levels of natural immunoglobulins and subsequently studied the animal’s immune response to antigenic challenges. DCs isolated from the spleens of HET RYR1Y522S mice express higher levels of the maturation marker CD83 compared to their wild type littermates, a result that is compatible with the finding that surface expression of CD83 is increased in mature human and murine DCs (Weissman et al., 1995; Zhou and Tedder, 1996; Berchtold et al., 1999; Kuwano et al., 2007). We have previously shown that in human DCs Ca\(^{2+}\) signals generated via RyR1 activation act synergistically with toll like receptors and induce DC maturation (Bracci et al., 2007) yet Stolk et al. (Stolk et al., 2006) reported that DCs isolated from WT and RyR1 knock out mice exhibit similar capacities to mature, endocytose antigens and stimulate T-cell proliferation. We would like to point out that chronic depletion of a protein from birth can activate compensatory mechanisms and the results of the present study support the role(s) of RyR1- signalling in DC function.
We are aware that the change in CD83 expression observed in DCs from HET RYR1\textsubscript{Y522S} mice is equivalent to a 30% increase, however (i) we exclude that such a small difference is due to differences in the genetic background of wild type and knock in mice since the mice were backcrossed in the same mouse strain (C57BL/6) and all experiments were performed on littermates, so that the only difference between the mice is a mutation in the \textit{RYR1} gene which causes increased agonist sensitivity and an increase in the cytoplasmic resting [Ca\textsuperscript{2+}] from approximately 50 nM to 125 nM, and (ii) the increase in CD83 expression is sufficient to cause a measurable change in function since in a mixed lymphocyte reaction, DCs from HET RYR1\textsubscript{Y522S} mice were more potent at stimulating T-cell proliferation and induced higher levels of IFN-\gamma release than was observed in T-cells stimulated with WT DCs. Since no increase in CD83 transcripts were observed, we suggest that the increase in CD83 surface expression in the HET RYR1\textsubscript{Y522S} mice is due to Ca\textsuperscript{2+} dependent release from an internal pool. Indeed increased surface expression of CD83 in DCs from WT mice could be induced already 2 minutes after the addition of the RyR1 agonist caffeine. These results are in line with the finding that CD83 molecules recycle between endosomes and the cell surface (Klein et al., 2005); in endosomes they co-localize with MHC class II molecules and we have previously shown that pharmacological stimulation of RyR1 results in the rapid expression of pre-formed MHC class II molecules on the cell surface (Vukcevic et al., 2008). Taken together these results support the observations that DC maturation results from the convergence of different signals arising from cytokine release, RyR1-activation and NFkB translocation (Bracci et al., 2007; Baeuerle and Henkel, 1994; Frantz et al., 1994).

An interesting observation concerning the role played by RyR1 in the immune system is that the sera of “un-stimulated” HET RYR1\textsubscript{Y522S} mice show elevated levels of circulating natural IgG\textsubscript{1} and IgE. Natural antibodies are produced in the absence of external antigenic stimulation and are directed against a wide spectrum of self- and non-self antigens (Avrameas et al., 2007); their role is related to early protection against pathogens (Ochsenbein et al., 1999; Baumgarth et al., 2005; McCoy et al., 2006). On the other hand, high levels of IgEs in conjunction with increased levels of eosinophils are often seen in the circulation of allergic individuals and are thought to arise because T cells orient towards the Th2 phenotype (Yazdanbakhsh et al., 2002). Our results suggest
that RyR1 signalling in DCs may affect Th-cell differentiation by influencing their
development into Th1 or Th2 and/or Treg. However, no changes in the release of IL-4
and TNF-α were observed in the mixed lymphocyte reaction and IL-10 and IL-4 were not
detected in the sera of either mouse group. Furthermore under basal conditions no shift in
the T-cell profile was observed in the spleens of WT and HET RYR1<sub>Y522S</sub> mice when
evaluated for expression of transcription factors and surface markers characteristic for
Th1 or Th2 cells. Accordingly, additional signals besides changes in Ca<sup>2+</sup> homeostasis
are probably required for T-cell orientation and release of IgG<sub>1</sub>/ IgE by plasma cells may
be controlled by several Th-dependent mechanisms.

**HET RYR1<sub>Y522S</sub> mice respond faster to antigenic challenges**

Our next question deals with the influence of altered calcium signalling on the
development of a specific humoral immune response. This was approached by
immunizing mice either with the non-infectious agent ovalbumin or, in light of the
elevated IgE levels, with the helminthic parasite *H. p. bakeri*. In both cases the levels of
antigen-specific IgG<sub>1</sub> reached similar levels in WT and HET RYR1<sub>Y522S</sub> mice at 2 weeks
or later; however, HET RYR1<sub>Y522S</sub> mice were more rapid at responding to antigenic
challenges during the early phases, i.e. at 7 days, indicating that the gain of function of
antigen presenting cells brought about by the *RYR1* knock in mutation, primes the
immune system so that it is more efficient at responding in a specific and T-cell
dependent way to antigenic challenges. Indeed anti-OVA IgM values were similar in WT
and HET RYR1<sub>Y522S</sub> mice. In the case of the parasitic infection HET RYR1<sub>Y522S</sub> mice
were also faster than WT littermates in responding to the infection and produced more
parasite-specific IgG<sub>1</sub> antibodies both to whole worm extracts and to the HES antigen,
even though they ultimately harboured the same level of parasite counts as their wild type
littermates. Vaccination with HES has been reported to confer protection to experimental
infection with *H. p. backeri*, but of the various antibodies generated, those recognizing
the VAL-1, VAL-2 and VAL-4 antigens are non-protective and possibly act as decoy
molecules giving rise to an ineffective immune response (Hewitson et al., 2011). In this
context it should be mentioned that the number of circulating eosinophils was similar in
both mice; thus, since eosinophils are involved in killing helminths, the early
development of specific IgG\textsubscript{1} is not sufficient to protect the host against this parasitic infection.

The main question underlying the experiments described in the present manuscript is whether or not gain of function mutations in the RyR1 Ca\textsuperscript{2+} channel offers, or not, a selective advantage to its host. The primary organ that is affected by mutations is skeletal muscle, and aside from rendering the muscle excitation-contraction machinery more excitable to low levels of stimulation, there seems to be no overt advantage. While investigating the relationship between MH and cytokines we found that the presence of MH-causing \textit{RYR1} mutations is associated with a higher release of the pro-inflammatory cytokines IL-6 and IL-1 from cultured myotubes and B-lymphocytes (Girard et al., 2001; Ducreux et al., 2004). Furthermore in humans, but not in mice, circulating levels of IL-1 and IL-6 are significantly elevated in individuals bearing dominant \textit{RYR1} mutations compared to control subjects (Treves et al., unpublished results), indicating a potential complex interplay between infection/inflammation/\textit{RYR1} mutations.

In conclusion, this report provides evidence that HET \textit{RYR1\textsubscript{Y522S}} mice carrying the \textit{RYR1} mutation Y522S have a gain in immune functions. These experiments represent “the proof of concept” that pharmacological activation of ”normal” RyR1 may enhance primary immune responses. These results also raise several potentially important scientific and medical issues, namely the possibility that individuals with a genetic predisposition to develop allergies, thus possessing a Th2 orientation (IgE, eosinophils, IL-4 and IL-10) may express gain of function polymorphic variants of \textit{RYR1}. Since vaccination induces a primary immune response and DCs represent a key target for adjuvant activity an important focus for future research in the field of new vaccine and adjuvant development could target the skeletal muscle RyR1 in doing so however, one must keep in mind that the RyR1 is predominantly expressed in skeletal muscle and its pharmacological activation could lead to severe consequences from abnormal muscle contraction to full-blown MH-like episodes.
MATERIALS AND METHODS

Mice: HET RYR1\textsubscript{Y522S} knock in mice model were kindly provided by Prof. Susan L. Hamilton (Chelu et al., 2006). Mice were caged in the Specific Pathogen Free SPF facility of the ZLF of the Basel University hospital and all experiments were performed following the regulations of the local Kantonal authorities (animal permit N° 1728 and 1729, 2081). For all experiments 8-10 week old male heterozygous (HET RYR1\textsubscript{Y522S}) mice and their wild-type littermate same sex siblings were used. Mice were genotyped by PCR amplification of genomic DNA isolated from a biopsy using the following primers and conditions: F 5’-TCT CCC TGG TCC TGA ATT GC-3’ and R 5’-AGC GTA CAG CCA CAC CAT TG-3’, 95°C 3 min followed by 30 cycles 95°C 30 sec, 54°C 45 sec, 72°C 45 sec and a final extension at 72°C for 4 min. Genotyping was performed by restriction enzyme digestion of the amplified genomic DNA with BplI restriction enzyme. DNA from WT mice (−/−) yields a band of about 700 bp, while digestion of the genomic DNA from HET RYR1\textsubscript{Y522S} mice (+/−) shows in addition to the band 700 bp, a band of 600 bp.

Isolation of DCs, T-cells and B-cells from mouse spleens: single-cell suspensions were prepared from mouse spleens by enzymatic disaggregation using collagenase type I (Sigma chemicals, St. Louis, MO, USA, C0130) according to the magnetic cell sorting protocol (Miltenyi Biotech, Bergisch Gladbach, Germany). DCs, T-cells and B-cells were isolated by positive sorting using anti-CD11c, CD4 and CD19-coated magnetic MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer’s instructions.

Resting [Ca\textsuperscript{2+}]: measurements were performed on fura-2 (Invitrogen, Basel, Switzerland) loaded CD11c\textsuperscript{+} splenic DCs isolated from wild type and HET RYR1\textsubscript{Y522S} knock in mice, as previously described (Ducreux et al., 2004). Briefly, after loading, cells were rinsed, resuspended in Krebs-Ringer medium containing 2 mM CaCl\textsubscript{2} and allowed to adhere to poly-lysine treated glass coverslips. Online (340 nm, 380 nm, and ratio) measurements were recorded using a fluorescent Axiovert S100 TV inverted microscope (Carl Zeiss, Jena, Germany) equipped with a 40 x oil immersion Plan NeoFluar objective (0.17 NA) and filters (BP 340/380, FT 425, BP 500/530) and attached to a Cascade 128+
CCD camera. Cells were analyzed using Metamorph and the average pixel value for each cell was measured at excitation wavelengths of 340 and 380 nm, as previously described (Ducreux et al., 2004). Fura-2 fluorescent ratio signals were converted into [Ca\(^{2+}\)] using the Fura-2 calcium imaging calibration kit from Molecular Probes (Invitrogen, Basel, Switzerland, catalogue N° F6774) following the manufacturer’s instructions. Images of Fura-2 in the different buffers were acquired using a 40 x oil immersion Plan NeoFluar objective (0.17 NA) and filters (BP 340/380, FT 425, BP 500/530) and attached to a Cascade 128+ CCD camera as described above for resting [Ca\(^{2+}\)] measurements.

**Flow cytometry:** was performed as previously described (Vukcevic et al., 2008) using a FACSCalibur equipped with Cell Quest software (Becton Dickinson Pharmingen, Basel, Switzerland). Briefly, cells were washed and resuspended in phosphate buffered saline (PBS), and incubated for 30 minutes at 4°C in the presence of fluorochrome labeled commercial monoclonal antibodies against the following surface markers: CD83, CD3, CD19, CD11c, CD11b, CD4, CD8, CD138, IgG1, IgM, IgE and I-Ab or isotype-matched controls (Becton Dickinson Pharmingen, Basel, Switzerland).

**RT PCR:** Total RNA was isolated from dendritic cells using RNAeasy kit from Qiagen and treated with deoxyribonuclease I (DNase I) (Invitrogen, Basel, Switzerland) to eliminate contaminant genomic DNA. After reverse transcription using 500 ng of RNA (high-capacity cDNA reverse transcription kit, Applied Biosystems, Forster City, CA, USA), cDNA was amplified by quantitative real-time PCR using SYBR Green technology (Fast SYBR Green Master Mix, Applied Biosystem, Forster Cita, CA, USA) and exon-intron junction-designed primers for TATA box binding protein, CD83, CD86, IL12p40 and IL23p19. Gene expression was normalized using self-TATA box binding protein as reference. The expression of RYR1 was investigated by semi-quantitative RT PCR. cDNA was amplified using primers and conditions as described in genotyping section. Amplified cDNA was digested with the restriction enzyme BplI; cDNA from WT mice (-/-) yields a band of approximately 376 bp, while digestion of the cDNA from HET RYR1Y522S (+/-) shows bands of approximately 376 bp and 276 bp plus a band of 100 bp.

**Mixed lymphocytes reaction assay and cytokine secretion:** mixed leukocytes cultures were set up in triplicate in 96-well flat-bottom microplates (Becton Dickinson
Pharmingen, Basel, Switzerland) with graded ratios of DC:T cells (1:2.5, 1:5, 1:10, 1:20, 1:40, 1:80, 1:160) using DCs isolated from WT and HET RYR1<sub>Y522S</sub> littermates with the C57BL/6 background as stimulator cells and 5x10⁴ T -cells as isolated from WT BALB/c mice (responder cells). Cultures were incubated for 4 days in RPMI medium supplemented with 10% foetal calf serum. During last 18h of culture, [³H]thymidine was added and lymphocyte proliferation was assessed by [³H]-thymidine incorporation. For cytokine secretion, the supernatants from mixed lymphocyte cultures were collected after 3 days of co-culture and the concentrations of IFN<sub>γ</sub>, TNFα, IL-4 and IL-10 were evaluated by ELISA (eBioscience, Inc, Becton Dickinson Pharmingen, Basel, Switzerland) following manufacturers’ instructions.

**Immunoglobulin Enzyme Linked Immunosorbent Assay (ELISA):** circulating levels of IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2b</sub>, IgG<sub>3</sub>, IgE and IgM were determined by a sandwich ELISA using goat anti-mouse IgG<sub>1</sub> (GeneTex Inc., catalogue number GTX29165), IgG<sub>2a</sub> (GeneTex Inc., catalogue number GTX29163), IgG<sub>2b</sub> (GeneTex Inc., catalogue number GTX29164) and IgG<sub>3</sub> (GeneTex Inc., catalogue number GTX77284) as coating antibodies and goat anti-mouse IgG peroxidase (Sigma, St Louis, MO, USA, catalogue number A2304) as a secondary Ab. In the case of IgE, plates were coated with goat anti-mouse IgE (SouthernBiotech, Birmingham, AL, USA, catalogue number 1110-01) and horse radish peroxidase conjugated goat anti-mouse IgE (SouthernBiotech, Birmingham, AL, USA, catalogue number 1110-05) were used for detection. As standards we used mouse IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2b</sub>, IgG<sub>3</sub> isotype controls (GeneTex Inc.) and mouse IgE isotype control (Biolegend, San Diego, CA, USA). To detect total IgM we used the ELISA kit from eBioscience (catalogue number 88-50470) and followed the manufacturer’s instructions. Sera were diluted from 1/10 to 1/50 000 depending on the immunoglobulin being investigated.

**Immunization and assessment of OVA specific IgGs:** wild type and HET RYR1<sub>Y522S</sub> knock in mice were immunized with 5 different concentrations of ovalbumin (10, 20, 50, 100 and 200 µg in 0.2 ml mixture of PBS and Alum as an adjuvant) and the sera were obtained 7, 14 and 21 days after the immunization. In some cases mice were re-immunized after 7 days. The presence of OVA specific IgG, IgG<sub>1</sub>, IgG<sub>2a</sub> and IgE antibodies was determined by ELISA. Briefly, 96-well plates were coated with 100µg/ml
OVA overnight at 4°C. Wells were washed with phosphate buffer saline (PBS), blocked with blocking buffer (1:100 dilution blocking solution from Roche in PBS) for 2 h at room temperature. Serum samples were diluted with PBS, added to the plates and incubated over-night at 4°C. Plates were washed with PBS, incubated with horse radish peroxidase conjugated secondary antibodies (goat anti-mouse IgG, IgG1, IgG2a and IgE diluted 1:4000 in PBS) for 1 hour at room temperature. The reaction was developed using 3,3',5,5' – tetramethylbenzidine followed by 0.16 M sulphuric acid as previously described (Ducreux et al., 2004) and the absorbance was monitored at 450 nm with a Synergy H1 ELISA reader (BioTek Instruments, GmbH).

*Heligmosomoides polygyrus bakeri* infection, worm count and measurements of IgGs against soluble extracts of whole worms and *H. p. bakeri* excretory-secretory (HES)

**Ag:** 8-10 week old WT or HET RYR1Y522S knock in mice were infected orally with 80 L3. Blood was taken 7 and 14 days after infection and blood cell counts were performed with an Advia 120 Haematology Analyzer using the Multispecies Software (Bayer, Leverkusen, Germany). For worm counts, mice were killed 14 days post-infection by CO2 euthanasia. For necroscopic examination, the entire intestine was removed from each mouse, placed in a Petri dish and opened longitudinally. All worms were removed and counted. Excretory-secretory antigens from adult *H. p. bakeri* (HES) were prepared as previously described (Hewitson et al., 2011). Soluble extracts of adult worms were prepared by lysing worms in 50mM HEPES pH 8.0, 1mM EDTA, 140 mM KCl 0.5% NP-40 1% Triton X-100, 1% Deoxycholate. After vortexing, the extracts were placed on a rotary shaker for 30 min shake and centrifuged at 10’000x g for 5 min. The supernatant (total soluble extract) was collected and the protein concentration measured using BCA protein assay kit according to the manufacturer’s instructions (Thermo scientific Inc).

Specific IgG and IgE antibodies in plasma were determined by ELISA in 96 well plates (COSTAR 9018) coated overnight at 4°C with 0.2 µg/well of total worm soluble extract or 0.4 µg/well of HES Ag in coating buffer (0.1M Carbonate/Bicarbonate buffer, pH 9.6). Wells were washed, blocked with blocking buffer for 2 h at room temperature and processed as described above for anti-OVA IgG determination.

**Blood samples:** blood was collected from WT and HET RYR1Y522S knock in mice in microtainer coated with EDTA (Becton Dickinson Pharmingen, Basel, Switzerland) and
diluted 1:4 in 0.9% Sodium Chloride solution; samples were analysed with an Advia 120 Haematology Analyzer using the Multispecies Software (Bayer, Leverkusen, Germany). Peripheral blood mononuclear cells were isolated from fresh blood by Ficoll density gradient centrifugation (Histopaque, catalogue number 1077, Sigma Chemicals, St Louis, MO, USA), washed once, resuspended in phosphate-buffered saline and incubated for 30 minutes at 4°C in the presence of fluorochrome labeled commercial monoclonal antibodies recognizing the following surface markers: CD4 and CD8 or isotype-matched controls (Becton Dickinson Pharmingen, Basel, Switzerland). The percentage of CD4+ or CD8+ cells was determined using a FACSCalibur instrument equipped with Cell Quest software (Becton Dickinson Pharmingen, Basel, Switzerland).

**T helper cell profiling:** Splenocytes were analyzed freshly or 5 days after stimulation (10^6 cells/ml) with CD3/CD28 coated Dynabeads (Invitrogen, Basel, Switzerland). For re-stimulation of T cells, Dynabeads were removed from the culture medium, cells were allowed to recover overnight and were then re-stimulated with CD3/CD28 coated Dynabeads in the presence of the protein transport inhibitor monensin (Becton Dickinson Pharmingen, Basel, Switzerland) for 6h. Prior to fixation, cells were stained for viability with the LIVE/DEAD cell staining kit (Invitrogen, Basel, Switzerland) and surface staining was performed. For intracellular detection of cytokines and transcription factors the Becton Dickinson (Pharmingen, Basel, Switzerland) Cytofix/Cytoperm Kit was used. Non-specific binding of Fcγ II/III receptors was blocked with anti-CD16/CD32 antibodies. Cells were analyzed on a FACSCanto II (Becton Dickinson Pharmingen, Basel, Switzerland). The following antibodies were used: phycoerythrin/Cy7 anti-mouse IL-4 and allophycocyanin anti-mouse IL-17A (Biolegend Europe), Alexa-Fluor 700 anti-mouse IFN-γ (Becton Dickinson Pharmingen, Basel, Switzerland); phycoerythrin anti-mouse GATA-3, peridinin chlorophyll protein anti-mouse T-bet, allophycocyanin anti-mouse Rorγt, phycoerythrin anti-mouse ICOS, phycoerythrin anti-mouse OX40 and phycoerythrin anti-mouse IL-13 (eBioscience Inc.).

**Statistical Analysis:** Statistical analysis was performed using the Student's *t* test; means were considered statistically significant when the P value was <0.05. When more than two groups were compared, analysis was performed by the ANOVA test followed by the Bonferroni post hoc test.
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Author contribution: M.V. designed and performed the experiments and analysed data; F.Z. helped design the experiments and analysed data; S.K. performed experiments and analysed data; D.A.T. designed experiments and analysed data, J.K. designed experiments and analysed data; R.M.M. designed experiments and analysed data; S.T. took care of conception and design of the experiments, collection and analysis of data, and drafting of the manuscript.

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Abbreviations:
DC, dendritic cells; ELISA; enzyme linked immunosorbent assay; HES Ag, H. pylori excretory-secretory antigen; HET, heterozygous; MHCII, Major Histocompatibility Complex Antigens class II; MH, Malignant Hyperthermia; MHS, Malignant Hyperthermia Susceptibility; OVA, ovalbumin; PBS, phosphate buffered saline; RyR, Ryanodine receptor, WT, wild type.
REFERENCES


FIGURE LEGENDS

**Figure 1:** Expression of RYR1<sub>Y522S</sub> in mouse CD11c<sup>+</sup> spleen DCs affects the resting [Ca<sup>2+</sup>]<sub>i</sub> and the expression of the maturation marker CD83. (A). Total RNA was extracted from purified DCs and the expression of RyR1 was evaluated by RT-PCR as described in the Methods section. Digestion of the RyR1 cDNA from wild type mice (WT) yields the uncut band of about 376 bp, while digestion of the cDNA from heterozygous mice (HET RYR1<sub>Y522S</sub>) yields two bands of 276 bp and 100 bp plus the uncut 376 bp band from the wild type allele. (B). The resting [Ca<sup>2+</sup>] of DCs from HET RYR1<sub>Y522S</sub> mice is significantly higher than that of their WT littermates (Student’s t test ***p<0.0001). Fluorescence measurements were performed on single DCs loaded with the ratiometric Ca<sup>2+</sup> indicator fura-2; results show the mean (± S.D.) intracellular free [Ca<sup>2+</sup>] (nM) of 74 WT (white bars) and 100 (HET RYR1<sub>Y522S</sub>, grey bars) cells. (C). CD11c<sup>+</sup> DCs were isolated from the spleens of WT and HET RYR1<sub>Y522S</sub> littermates and the % positive CD83 DCs was determined by flow cytometry; the % CD83 positive DCs is significantly higher in the RYR1<sub>Y522S</sub> mice (Student’s t test *P<0.01). The inset shows that treatment of DCs from WT mice with 10 mM caffeine significantly increases surface CD83 expression. Results represent the Mean Fluorescence Intensity values from 4 mice performed in duplicate (ANOVA followed by Bonferroni’s post hoc test **P < 0.0001; *** P<0.0000002). (D). Real time PCR for the indicated maturation markers were performed on DCs from WT (empty bars) and HET RYR1<sub>Y522S</sub> (grey bars) mice. Boxes represent the mean (±s.e.m.) fold increase of 4 different experiments carried out on CD11c<sup>+</sup> cells from different mice. Results were not statistically different.

**Figure 2:** DCs from HET RYR1<sub>Y522S</sub> mice are more efficient at stimulating T cells than their WT counterpart as assessed by the mixed lymphocytes reaction. (A). DCs were isolated from WT (white bars) and HET RYR1<sub>Y522S</sub> mice (grey bars) (C57BL/6) and cultured with allogenic CD4<sup>+</sup> T cells isolated from Balb/c mice as detailed in the methods section, in graded DC to T cells ratios. Bars indicate mean (±s.e.m.) [³H]thymidine incorporation from three different experiments carried out in triplicate.
(B). Analysis of cytokines released into the supernatant following the mixed lymphocyte reaction at a DC: T cell ratio of 1:2.5 as detailed in the methods section. T-cells incubated with HET RYR1_{Y522S} DCs (grey bars) release significantly higher levels of IFNγ compared to T-cells incubated with WT DCs (empty bars). Results are the mean (± s.e.m.) of 3 experiments carried out in triplicate.

**Figure 3:** Pre-immune serum IgG1 and IgE levels are significantly higher in HET RYR1_{Y522S} knock in mice compared to their wild type littermates. Mice were caged in the specific pathogen free facility of the ZLF of the Basel University Hospital; each point represents the concentration of the indicated immunoglobulin determined per mouse. WT (black boxes) and HET RYR1_{Y522S} knock in mice (black triangles). P was significantly different according to the Student’s t test.

**Figure 4:** HET RYR1_{Y522S} knock in mice produce higher titres of antigen-specific IgG1 than their wild type littermates at the early stages post-infection/immunization. (A) Mice were immunized with 100 µg OVA as described in the methods section and the concentration of circulating OVA-specific IgG1 were determined after 7, 14 and 21 days. After 7 days the mice were re-immunized. Each point represents the average level of IgG1 per mouse. WT (black boxes) and HET RYR1_{Y522S} knock in mice (black triangles). The serum levels of anti-OVA IgG1 are significantly higher in the HET RYR1_{Y522S} mice. Statistical analysis was performed using the ANOVA test followed by the Bonferroni post hoc test (*P<0.02). (B) Mice were orally infected with *H. p. bakeri* larvae and the concentration of worm-specific (left panel) or HES-specific (left panel) IgG1 were determined after 7 by ELISA. Each point represents the average amount of IgG1 per mouse. WT (black boxes) and HET RYR1_{Y522S} knock in mice (black triangles). P was significantly different according to the Student’s t test.
**A**

Anti-OVA IgG1

- **7 days**: P < 0.02
- **14 days**: no significant difference
- **21 days**: no significant difference

**B**

IgG1 anti-H. p. bakeri 7 days

- WT: O.D. (450 nm) range
- HET RYR1<sub>Y522S</sub>: O.D. (450 nm) range

P < 0.035

IgG1 anti-HES 7 days

- WT: O.D. (450 nm) range
- HET RYR1<sub>Y522S</sub>: O.D. (450 nm) range

P < 0.04