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Crucial role for immune complexes but not FcRn in immunization against anti-TNF- $\alpha$   
antibodies after a single injection in mice

Running title: Immune complexes and immunization against mAbs

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## **Abstract**

Immunogenicity of infliximab and adalimumab is a major concern, since patients may develop Abs also called Anti-drug Abs (ADA), directed against these anti-TNF- $\alpha$  after only a few weeks of treatment. These ADAs can lead to a decrease in biologic concentration, which was associated with lower treatment efficacy. Our aim was to study the involvement of immune complexes (ICs) and neonatal Fc receptor (FcRn) in the emergence of anti-drug-antibodies (ADAs) in the case of anti-TNF- $\alpha$  antibodies. Wild type and FcRn KO mice were injected once with either infliximab or adalimumab, alone or pre-incubated with TNF- $\alpha$ . Adalimumab cross-reacts with murine TNF- $\alpha$  whilst infliximab is species-specific. When injected alone, only adalimumab elicited a humoral response. By preforming immune complexes with TNF- $\alpha$ , an anti-infliximab response was elicited. Surprisingly, both wild type and FcRn KO mice were able to mount an immune response against anti-TNF- $\alpha$  antibodies suggesting that ICs are a major determinant of this immunization.

## Introduction

Anti-TNF- $\alpha$  antibodies (Abs) are widely used, and some of them are even blockbuster biopharmaceuticals. Anti-TNF- $\alpha$  Abs, like adalimumab or infliximab are indicated in the case of severe rheumatoid arthritis, in first-line treatment or in mild cases when disease-modifying antirheumatic drugs or methotrexate alone have failed to control disease progression (1, 2). Yet, immunization issues leading to inter-individual variability tarnish their success, frequently forcing clinicians to switch to another treatment line.

Immunogenicity of infliximab and adalimumab is a major concern, since at least 14% and up to a third of the patients develop Abs also called Anti-Drug Abs (ADA), directed against these anti-TNF- $\alpha$  after only a few weeks of treatment (3, 4). These ADAs can lead to adverse effects during infusion or to a decrease in biologic concentration, which was associated with lower treatment efficacy (4).

Many factors have been suspected to be involved in the immunization against therapeutic antibodies. Even though some were refuted, like the degree of Ab humanization (5), or discussed, like the IgG allotypes (6–8), several other factors were showed to be involved, like dosing and administration schedule, under-exposure to the anti-TNF- $\alpha$  drug(9), co-administration of other drugs (10) and finally formation of aggregates (11). Complexes can be formed between several molecules of the therapeutic Ab (aggregates) or with its target antigen as in classical immune complexes (ICs) to constitute target-related ICs (TRICs) (12). It has been shown that monoclonal Abs (mAbs) are immunogenic *in vivo* when they form TRICs (13). This phenomenon can be understood in the light of the recent literature concerning IC presentation by immune cells via the neonatal Fc receptor (FcRn) and with the work of Foss *et al.* who recently demonstrated that TRICs are more efficiently transcytosed than monomeric mAb *via* FcRn (14). These mechanisms bring into play the well described

functions of FcRn; extension of IgG half-life, IgG biodistribution and involvement in humoral and anti-tumoral immune response through IC presentation by immune cells (15, 16).

The present work focuses on the ability of TRICs to trigger a primary immune response (*i.e.* after a single injection) against anti-TNF- $\alpha$  antibodies in the presence or absence of FcRn. In order to answer this question, anti-TNF- $\alpha$  antibodies were administered to wild type and FcRn knock out mice and the formation of ADA was measured. The first one, infliximab specifically recognizes hTNF- $\alpha$  whilst the other one, adalimumab cross-reacts with mTNF- $\alpha$ , allowing to evaluate the role of TRICs and FcRn in ADA formation.

## **Materials and methods**

### *Antibodies*

The following mAbs were used: infliximab and golimumab (anti-TNF- $\alpha$  IgG1, MSD), adalimumab (anti-TNF- $\alpha$  IgG1, Abbvie) and rituximab (anti-CD20 IgG1, Roche).

### *Administration of anti-TNF- $\alpha$ mAb in wild type (WT) and *fcgrt*<sup>-/-</sup> (FcRn KO) mice*

Six- to eight-week-old C57BL/6 WT mice were obtained from Janvier® (Saint-Berthevin, France). The B6.129X1-Fcgrttm1 Dcr/DcrJ (*fcgrt*<sup>-/-</sup>) were generated by the Jackson Laboratory (Bar Harbor, Maine, USA). A targeting vector was designed to replace 1588 nucleotide fragments (encoding promoter sequence 5' of the transcriptional start site, exon 1, intron 2, and most of exon 2) with a PGK-Neor cassette. The vector was electroporated into 129X1/SvJ-derived ESV/J-1182 embryonic stem (ES) cells. Correctly targeted ES cells were injected into recipient C57BL/6J blastocysts. The resulting chimeric animals were crossed to C57BL/6J mice. The mice were then backcrossed to C57BL/6J for 11 generations. All experiments were performed in accordance with national animal care guidelines (EC directive 86/609/CEE, French decree n°87-848), and were approved by the Val-de-Loire Animal

Experiments committee of ethics (approval n° 2012-04-09). Body weight was monitored throughout the study, as an indicator of health status. After one week of adaptation period in a same room, WT and FcRn KO mice were anesthetized with isoflurane 2.5% and intravenously injected by retro-orbital route, with a single dose of 4mg/kg adalimumab or infliximab. Blood samples were collected at 2 and 6h, and on days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13, 14 and 21 after adalimumab or infliximab injection. Blood samples were centrifuged at 1500×g for 15 minutes, and sera were stored at -20°C until measurement of infliximab, adalimumab or ADA levels.

*Injection of hTNF- $\alpha$ /anti-TNF- $\alpha$  mAb immune complexes in WT and FcRn KO mice.*

ICs were generated *in vitro* by mixing anti-TNF- $\alpha$  Ab and hTNF- $\alpha$  (Peprotech, Neuilly-sur-Seine, France), respectively in a 1:2 molar ratio, and left for 1h at room temperature. The final concentrations in PBS were 500 $\mu$ g/mL for anti-TNF- $\alpha$  Ab and 116 $\mu$ g/mL for hTNF- $\alpha$ . Two hundred  $\mu$ L were intravenously injected by retro-orbital route. Blood samples were collected before mAb injection and on days 5, 7, 10 and 21 for infliximab and adalimumab, or on day 3, 10 and 20 for golimumab.

*Pharmacokinetic (PK) analysis*

The serum concentrations of infliximab and adalimumab were measured using validated ELISA techniques (17, 18). Briefly, recombinant human TNF- $\alpha$  was coated on the solid phase and recognized by infliximab or adalimumab, and the therapeutic antibody was detected by an anti-human IgG Fc $\gamma$ -specific antibody conjugated to horseradish peroxidase.

### *ADA and TNF- $\alpha$ ELISA*

Ninety six flat-bottomed plates were coated with one  $\mu$ g per well of adalimumab, infliximab or golimumab in 0.1M NaCO<sub>3</sub>/NaHCO<sub>3</sub> buffer (pH9.6) in case of ADA ELISA or with 200ng per well of human or murine TNF- $\alpha$  (Peprotech, Neuilly-sur-Seine, France) in TNF- $\alpha$  ELISA. The plates were incubated 12h at 4°C. Washing steps were performed with PBS, 0.1% tween 20, plus 5% casein in blocking step or in mAb dilutions. Serum dilutions (1:100 unless otherwise specified) were incubated at room temperature for 1h. Specific binding was detected by using either peroxidase-labeled donkey anti-mouse IgG (H+L) (Jackson laboratory, Bar Harbor, Maine, USA) for 1h at room temperature in ADA ELISA, peroxidase-labeled goat anti-human IgG (H+L), peroxidase-labeled anti-IgG antibody (anti-Fc, Jackson laboratory, Bar Harbor, Maine, USA) or with a peroxidase-labeled anti-IgM antibody (anti-Fc, Jackson laboratory, Bar Harbor, Maine, USA). The reaction was developed by adding 100 $\mu$ L of tetramethylbenzidine (Sigma Aldrich, Saint Louis, Missouri, USA) for 20min and stopped with 50 $\mu$ L H<sub>2</sub>SO<sub>4</sub> 0.5M. Absorbance was measured at 450nm with a Mithras LB 940 Multimode Microplate Reader (Berthold Technologies, Thoiry, France).

### *Flow cytometry analysis.*

Mice spleens were analyzed 2 or 6 days after Ab injection. Single-cell suspensions were isolated with Spleen Dissociation Kits (Miltenyi Biotec, Paris, France). Total leukocyte cell counts were performed and  $1 \times 10^6$  cells were analyzed by flow cytometry. Fc $\gamma$ R were first saturated by cell incubation with an anti-Fc $\gamma$ RII/Fc $\gamma$ RIII mAb (2.4G2, BD Pharmingen, San Diego, CA) for 20min. Then, cells were incubated with fluorochrome-conjugated specific Abs, in PBS containing 2% SVF and 0.1% sodium azide at 4°C for 60min. Cells were analyzed using a FACS Gallios™ equipped with Kaluza software (Beckman Coulter, Villepinte, France). Anti-mouse CD3-FITC, Ly-6c PE-CF594, CD11c PerCP-Cy5.5,

CD45R/B220 PE-Cy7, CD11b APC-Cy7 and corresponding control isotypes were purchased from BD Pharmingen (San Diego, CA).

### *Statistical analysis*

Flow cytometry and ELISA data are expressed as mean and standard deviation of independent experiments. The Mann-Whitney test was used to determine significant differences unless otherwise specified. Statistical analysis was performed using GraphPad Prism 5. The alpha risk was set at 5%. The level of significance is indicated on the figures as \* for  $p \leq 0.05$ , \*\* for  $p \leq 0.01$  and \*\*\* for  $p \leq 0.001$ .

## **Results**

### *Adalimumab and infliximab have different elimination profiles in WT mice*

To measure the relation between anti-TNF- $\alpha$  mAb concentrations and the formation of ADA, adalimumab and infliximab concentrations were monitored during 21 days in the serum of WT and KO mice after a single 4 mg/kg mAb injection (Figure 1). As expected, serum concentrations of both mAbs were undetectable after 7 days in FcRn KO mice. An acceleration of mAb elimination, with a nonlinear decrease, was observed especially for adalimumab in WT mice, suggesting the formation of ADA.

### *ADA follow-up formation reveals immunization against adalimumab but not infliximab in both WT and FcRn KO mice*

ADA formation against adalimumab or infliximab was monitored in both WT and FcRn KO mice after a single mAb injection. The results, presented in figure 2, show that ADA are detected earlier in FcRn KO than in WT mice after adalimumab injection (day 4 and day 6 respectively). Even though ADAs occurred earlier in FcRn KO mice, the titers obtained in



WT mice seem to be higher (Figure 2C). The two main mechanistic explanations for this phenomenon are a booster role of FcRn in the humoral response, leading to increased Ab production in WT mice, and the protection of these IgGs from lysosomal degradation by FcRn. ADA detection with an anti-mouse-IgG-specific or an anti-mouse-IgM-specific antibody revealed that the response is mainly an IgG response with very low IgM levels. By contrast, infliximab did not induce any ADA response up to 21 days after injection in both WT and FcRn KO mice (data not shown).

#### *TRICs are involved in ADA development*

Our hypothesis was that infliximab did not induce ADA due to the lack of TRIC formation with the endogenous mTNF- $\alpha$ , whilst adalimumab cross-reacts with mTNF- $\alpha$  and therefore generates TRICs following injection. First, the species-specificity of anti-TNF- $\alpha$  mAbs was assessed. As shown in figure 3, adalimumab binds both human and murine TNF- $\alpha$  while infliximab only binds human TNF- $\alpha$ . Rituximab, an anti-CD20 mAb, was used as negative control. Then, infliximab-containing TRICs were constituted *in vitro* by mixing human TNF- $\alpha$  and infliximab prior to injection to WT and FcRn KO mice. No ADA could be detected by ELISA in WT or FcRn KO mice injected with infliximab alone. On the contrary, high amounts of infliximab ADA were measured in WT as well as in FcRn KO mice injected with *in vitro*-formed infliximab-containing TRICs (figure 4). No anti-TNF- $\alpha$  Abs were detected in mice injected with recombinant human TNF- $\alpha$  alone. Altogether these data showed that artificially formed TRICs are able to elicit immune response to infliximab. In FcRn KO mice, ADA formation has different kinetics depending on the injected mAb with anti-adalimumab Abs appearing more rapidly than anti-infliximab Abs, whilst ADA decreased more quickly in FcRn KO mice than in WT mice for both mAbs. We injected a 10 fold higher concentration of adalimumab (40mg/kg), and showed that immunization occurred

later in these mice, suggesting that dilution of the immune complexes in a tide of uncomplexed antibodies delays ADA formation.

*Degree of humanization is not responsible for adalimumab immunogenicity*

The lack of immunogenicity of infliximab alone could be due to the fact that its variable domains are murine while adalimumab has human variable domains. To address this question, a new set of experiments was performed with golimumab, a fully-human therapeutic anti-TNF- $\alpha$  mAb, which is specific to human TNF- $\alpha$ , as shown in figure 5A. Golimumab was injected once, either alone or after *in vitro* IC generation by incubation of golimumab with human TNF- $\alpha$ . Golimumab alone did not elicit ADA response in WT mice, in contrast to preformed golimumab-TRICs (Figure 5B). These results were confirmed by repeating the experiment and performing ELISA with an anti-IgG HRPO-labeled murine-Fc $\gamma$ -specific antibody (data not shown).

*TRICs induced a cellular immune response in both WT and FcRn KO mice*

Spleens of WT and FcRn KO animals injected with adalimumab were harvested on day 2 or day 6 in order to examine the cellular population. Spleens from naive animals were used as control (“day 0” in figure 6). As showed in figure 6A, the size of the spleen increased 2 days after adalimumab injection with a largely more pronounced effect in FcRn KO mice. This phenomenon was described in all mice by total splenocyte count (figure 6A), and a return to a normal count was observed on day 6. Flow cytometry analysis revealed that the increase in cell count on day 2 after adalimumab injection was observed for B cells (B220+), T lymphocytes (CD3+), dendritic cells (Ly6c+/CD11b+) and neutrophils (CD11c+/CD11b+) with a 1.43 to 1.97 ratio (day 2/day 0), compiling all FcRn KO and WT mice data (figure 6B). As expected, ratios were higher in FcRn KO (1.73 to 2.24) as compared to WT mice (1.40 to 1.57) for all cell types. This increase in cell count was more pronounced for B cell ratio (2.24

in FcRn KO versus 1.45 in WT mice), and dendritic cells (1.97 in FcRn KO versus 1.40 in WT mice), followed by a milder increase in neutrophils (1.84 in FcRn KO versus 1.43 in WT mice) and T cells (1.73 in FcRn KO versus 1.57 in WT mice).

## Discussion

The immunogenicity of therapeutic mAbs is a complex phenomenon that can occur during treatment with therapeutic mAbs leading to the formation of ADA-related ICs (ARICs) and a profound alteration in mAb pharmacokinetics in most patients (9, 19, 20). These ARICs are defined as complexes formed by the binding of ADA to mAbs, particularly to the mAb idiotypes, since paratopes remain immunogenic whatever the degree of humanization of mAbs (5). This phenomenon has been evidenced in patients treated with infliximab or adalimumab and measurement of ADA is often a warning sign of upcoming treatment failure after an initial response (9, 21, 22). In order to prevent immunization, it is necessary to understand the mechanisms leading to the formation of ADA. Our study shows that TRICs but not FcRn are essential to trigger an immune response (*i.e.* after a single injection) against anti-TNF- $\alpha$  antibodies. Moreover, although the crucial role played by FcRn in the development of humoral immune responses has been demonstrated by several studies (23, 24), our data fit better with a booster role for FcRn (25). Most of these studies being carried out using ovalbumin because the T-epitopes are well characterized and can be followed-up, the specific case of anti-TNF- $\alpha$  antibodies is different. In our study, the antigens are antibodies which form TRICs that elicit an immune response after the first injection (or not in the case of infliximab). This point could modify the kinetics of the immune response. In our animal model, we first confirmed that the presence of ADA modifies the pharmacokinetics of therapeutic antibodies. Adalimumab displayed a faster log-linear elimination than infliximab but, above all, an accelerated terminal elimination, characteristic of the development of ADA

(26, 27). This acceleration was observed in all mice treated with adalimumab, independently of the FcRn status even if elimination was clearly faster in FcRn KO mice (28). Adalimumab binds mouse TNF- $\alpha$  as described in the European Medicine Agency document (29) and confirmed by ELISA. We hypothesized that with adalimumab treatment TRICs would be formed and could in turn cause immunization. To address this question, we incubated infliximab and TNF- $\alpha$  to form TRICs, which were able to induce anti-infliximab Abs after injection in both WT and FcRn KO mice.

The antibodies used differ in several ways, and one could wonder whether the results could be biased due to confounding factors. Adalimumab is a fully human therapeutic mAb. In order to discard the possibility that immunization could be a result of the presence of human portions in the protein sequence, we performed similar experiments with golimumab, another fully human anti-TNF- $\alpha$  mAb very specific for human TNF- $\alpha$ . The absence of anti-golimumab Abs supports the idea that mAb humanization is not responsible in the development of ADA in our model. However, as observed with infliximab, golimumab-containing TRICs injected to mice were able to elicit an anti-golimumab response. Apart from the degree of humanization, allotypes have been suspected to participate in the immunization process in chronic inflammatory diseases, even though the results were negative (6). All the antibodies used here shared the same allotype (G1m17; 1), which discards this hypothesis. Finally, the intrinsic characteristics of the TRICs themselves, such as their size (25), are involved in their trafficking and condition the type of immune response elicited (30). But since FcRn<sup>-/-</sup> mice also mount an immune response against these ICs discards this possibility. Moreover, infliximab incubated with hTNF- $\alpha$  also elicits an immune response. Altogether, these data confirmed that TRICs are necessary to elicit a primary immune response in the case of anti-TNF- $\alpha$  antibodies.

FcRn is involved in the recycling and transcytosis of IgGs and monovalent immune complexes (31). But surprisingly, ADAs were detected in both WT and KO FcRn mice suggesting that FcRn is not necessary for immunization against anti-TNF- $\alpha$  Abs. Even more, anti-adalimumab Abs appeared more rapidly in FcRn KO mice than in WT animals. Several studies performed in FcRn KO models show that mAb pharmacokinetics is clearly impaired and that IgG biodistribution is differently affected depending of the organ tested (28, 32). We hypothesized that, in absence of FcRn, adalimumab would not be recycled and that, its biodistribution outside the vascular compartment being altered, it would be fully available to form ICs with its target, especially in places such as mesenchymal lymph nodes, where ADA concentrations remain sufficient to form TRICs due to resident plasma cells as described by Baker et al. (16). TRICs would then be able to rapidly induce an immune response, characterized by IgG and low IgM ADAs, probably via Fc $\gamma$ R binding and MHC class II presentation (31, 33, 34). Another non-exclusive hypothesis is that the concentrations of free adalimumab are lower in FcRn<sup>-/-</sup> mice, and that their tolerogenic effect over TRICs is therefore shorter. Although dependence of Ab-mediated presentation of Ag on FcRn has been described in the case of secondary humoral response (15, 25), its absence could probably be overcome by other molecules for IC routing and MHC class II presentation as suggested in our model. The ADA response has a cellular component as observed by the increase in dendritic/monocyte counts as well as B or T-cell counts in the spleen of immunized animals. This cellular immune response is necessary to lead to the humoral response and is proportional to the amount of ADA generated. Chen *et al.* have shown that in the liver or the spleen, FcRn may exert its transcytosis function to transport IgGs out of the tissue (28). In the absence of FcRn, mAbs/TRICs might be retained in the spleen, thus participating in the increased immune response. Simultaneously, the absence of FcRn leading to accelerated clearance of adalimumab could also explain the faster clearance of ADA in FcRn KO mice as

compared to WT. The involvement of this mechanism in ADA kinetic differences is sustained by the kinetics observed with preformed TRICs in our model. In this case, anti-infliximab Ab formation occurs without delay. The question remains as to which FcγRs participate in this immune response. The cellular counts performed on the harvested spleen show an increase in multiple cell populations 2 days after the injection of preformed TRICs among which dendritic cells (CD11c+/CD11b+), neutrophils and monocytes (LY6c+/CD11b+). These cells express the activating murine FcγRI (monocytes, dendritic cells), FcγRIII and FcγRIV (dendritic cells, neutrophils and monocytes) that could all play a role in this process. However, because of the numerous differences between humans and mice in terms of FcγR,(35) caution must be used for extrapolation to the clinical situation.

In humans, ADAs have been detected in a large number of patients treated with anti-TNF-α mAbs. Under-exposure to mAbs has been clearly demonstrated in patients as a risk factor of immunization (19, 20). This approach does not take into account the evaluation of specific TRICs, which are known to skew the dosage of ADAs in the serum (36). The nature of the target may also participate to the formation of ICs. In that context, TNF-α is a good candidate since it is composed of a trimer and exists as soluble Ag that is more prone to form multimeric ICs. The three antibodies used here target TNF-α in different ways. Adalimumab and infliximab stabilize TNF-α as a trimer, while golimumab binding does not prevent monomer exchange with unbound trimers (37), which could explain the rather important variability observed in the anti-golimumab responses. This could explain the higher levels of ADA found with anti-TNF-α than with biologics directed against other molecules, along with the inflammatory profile of the pathologies involving high titers of TNF-α.

It is important to bear in mind that this study was performed using FcRn KO mice, meaning that the bioavailability of the TRICs is altered compared to WT mice, and that full FcRn deficiency has never been evidenced so far in humans.

It has been documented that low concentrations of anti-TNF- $\alpha$  antibodies is associated with increased ADA formation in patients (21), and hypothesized that maintaining high concentrations of monomeric antibody could prevent the immunization process (12). In the case of anti-TNF- $\alpha$  therapy and primary immune response in mice, we show that this is true.

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## Disclosure

The authors declare that the research was conducted in the absence of any financial relationships that could be construed as a potential conflict of interest.

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8 2- Abbreviations used in this article: FcRn: neonatal IgG Fc receptor; mAb: monoclonal  
9 antibody; ADA: Anti-drug Abs; ICs: immune complexes; TRICs: target-related ICs; WT  
10 mice: wild type mice; FcRn KO mice: *fcgrt*<sup>-/-</sup> mice.

11

## Figure legends

**Figure 1:** Pharmacokinetics of infliximab and adalimumab after a single 4 mg/kg injection. Median serum concentrations over time for adalimumab (triangles) and infliximab (circles) in WT mice (black) and KO mice (grey) for each antibody. Each data set (n=7 per group) corresponds to mean  $\pm$  SEM.

**Figure 2:** Detection of ADA against adalimumab and infliximab in WT (black lines) and FcRn KO (grey lines) mice. ADA formation was evaluated by blood sampling over three weeks and measured by ELISA in the serum. Each data set corresponds to mean  $\pm$  SEM of absorbance values in arbitrary units (AU).

A. Detection of IgM ADA against adalimumab in WT (n=7) and FcRn KO mice (n=8). ADA formation was evaluated by blood sampling over three weeks and measured by ELISA in the serum (serum dilution 1:100). B. and C. Detection of IgG ADA against adalimumab in WT (n=7) and FcRn KO mice (n=8). ADA formation was evaluated by blood sampling over three weeks and measured by ELISA in the serum, at two different serum dilutions (1:100 for B and 1:1000 for C). D. Detection of IgG ADA against adalimumab at 4mg/kg in WT (n=7) and FcRn KO mice (n=8) or at 40mg/kg in WT mice (n=7). ADA formation was evaluated by blood sampling over three weeks and measured by ELISA in the serum (diluted at 1:100). P-values are represented as follows: \* for  $p < 0.05$ , \*\* for  $p < 0.01$  and \*\*\* for  $p < 0.001$ .

**Figure 3:** Analysis of anti-TNF- $\alpha$  mAb specificity by ELISA. Adalimumab and infliximab were tested in a human and murine TNF- $\alpha$  ELISA. The anti-CD20 rituximab was used as a negative control. Results of three independent experiments are presented as mean  $\pm$  SEM of absorbance values in arbitrary units (AU). P-values are represented as follows: \*\*\* for  $p < 0.001$ .

37

38 **Figure 4:** *In vitro*-formed infliximab containing TRICs leads to infliximab ADA  
39 development. WT and FcRn KO mice were injected either with the anti-TNF- $\alpha$  mAb alone  
40 (solid lines) or with *in vitro*-formed infliximab containing TRICs (dashed lines). Infliximab  
41 ADA were measured in the serum of mice (4 mice per group) at indicated times after  
42 injection. The results correspond to mean  $\pm$  SEM of absorbance values in arbitrary units  
43 (AU). On day 21, there is no statistically significant difference in the ADA titer between WT  
44 or FcRn-KO mice injected with pre-formed ICs ( $p=0.056$ ).

45

46 **Figure 5:** A) Analysis of golimumab specificity for human TNF- $\alpha$  by ELISA. B) Injection of  
47 WT mice with golimumab alone or with *in vitro*-formed TRICs with golimumab and human  
48 TNF- $\alpha$ . Golimumab ADA were measured in the serum of mice (4 mice per group) at  
49 indicated times after injection. The results correspond to mean  $\pm$  SEM of absorbance values  
50 in arbitrary units (AU). P-values are represented as follows: \* for  $p < 0.05$ , and \*\* for  $p <$   
51  $0.01$ .

52

53 **Figure 6:** Cell content analysis of WT and KO FcRn mice spleens 2 days (d2) and 6 days (d6)  
54 post-adalimumab injection by flow cytometry. Cells harvested from naïve animals represent  
55 the time point d0. A) Spleens from adalimumab-treated WT and FcRn KO mice are presented  
56 after two days of treatment (d2) and compared to spleens from naïve mice (d0). Total  
57 splenocyte cell count was analyzed in 4 mice and results correspond to mean  $\pm$  SEM. B)  
58 CD3<sup>+</sup> T lymphocytes, B220<sup>+</sup> B lymphocytes, CD11b<sup>+</sup>/CD11c<sup>+</sup> dendritic cells and  
59 Ly6c<sup>+</sup>/CD11b<sup>+</sup> monocytes/neutrophils were estimated by specific cell count in 4 mice and  
60 results corresponds to mean  $\pm$  SEM. P-values are represented as follows: \* for  $p < 0.05$ .

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