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RESEARCH PAPER

The Immunogenicity of Antibody Aggregates in a Novel Transgenic Mouse Model

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ABSTRACT

Purpose Protein aggregates have been discussed as a potential risk factor related to immunogenicity. Here we developed a novel human IgG transgenic (tg) mouse system expressing a mini-repertoire of human IgG1 antibodies (Abs) for the assessment of immunogenic properties of human mAb preparations.

Methods Transgenic mice were generated using germline versions of the human Ig heavy chain γ1 (IgH-γ1), and the human Ig light chain (IgL) κ and λ genes. Only the soluble form of human IgH-γ1 was used to avoid expression of the membrane Ig-H chain and concomitant allelic exclusion of endogenous murine Ig genes. IgG1 aggregates were generated by different stress conditions such as process-related, low pH and exposure to artificial light.

Results The expression of human Ig proteins induced immunological tolerance to a broad range of human IgG1 molecules in the tg mice. Immunization with IgG1 aggregates demonstrated that soluble oligomers induced by significant light-exposure and carrying neo-epitopes induced a strong immune response in tg mice. In contrast, Ab aggregates alone and monomers with neo-epitopes were not immunogenic.

Conclusion This mouse model is able to recognize immunogenic modifications of human IgG1. While the degree of stress-induced aggregation varies for different mAbs, our findings using a particular mAb (mAb1) demonstrate that non-covalently modified aggregates do not break tolerance, contrary to widely held opinion. The immunogenic potential of soluble aggregates of human IgG strongly depends on the presence of neo-epitopes resulting from harsh stress conditions, i.e. extensive exposure to artificial light.

KEY WORDS anti-drug antibodies · human IgG transgenic mouse · immune tolerance · therapeutic antibodies aggregates

ABBREVIATIONS

Ab	Antibody
ADA	Anti-drug-antibodies
AFCs	Antibody forming cells
APC	Antigen-presenting cells
BM	Bone-marrow
DCs	Dendritic cells
FCM	Flow cytometry
h	Human
H	Ig heavy chain
HMW	Higher molecular weight
IC	Immune complex
Ig	Immunoglobulin
KLH	Keyhole limpet hemocyanin
L	Ig light chain
LC/MS	Liquid-chromatography/mass spectrometry

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mAb(s)	Monoclonal antibody(ies)
MAPPs	MHC-associated peptide proteomics
met	Methionine
MZ	Marginal zone
s.c.	Subcutaneous(ly)
SEC	Size exclusion chromatography
tg	Transgen(ic)
V	Variable region

INTRODUCTION

Human monoclonal antibodies (mAbs) are effective therapeutic agents and their clinical applications have significantly grown during the past years (1, 2). Most therapeutic mAbs, being humanized or fully human proteins, are not expected to induce an immune response in recipient patients. However, humoral responses, resulting in anti-drug antibodies (ADAs), may occur in patients treated with mAbs or therapeutic proteins (3–5). The causes of ADAs induction may be patient-, disease- or product-related (6). Protein aggregates are one of the product attributes that have been discussed in the recent literature as a potential risk factor related to immunogenicity (7, 8). The clinical consequences of immunogenicity in treated patients vary widely (9, 10). The induced ADAs may be innocuous or at worst they may cause severe side effects due to neutralization of endogenous proteins, as observed with recombinant erythropoietin-alpha (11). Models are therefore beneficial in order to assess the immunogenic potential of therapeutic protein preparations. Among these tools, *in silico*, *in vitro* and *in vivo* approaches are currently being explored (12). While *in silico* and *in vitro* methods assess e.g. the conditions for T-cell activation, transgenic (tg) *in vivo* systems comprise the entire immune system and therefore may better simulate the multi factorial cascade which results in ADA responses. But, because human therapeutic proteins are generally immunogenic in experimental animals, animal experiments are hardly predictive of immunogenic potential in humans, making clinical studies finally required.

To study these questions, few human Ig tg mouse models have been applied (13, 14). Here, we developed a transgenic (tg) mouse model to facilitate the experimental *in vivo* assessment of immunogenicity of therapeutic Abs subjected to three different stress conditions. This model relies on the status of immune tolerance to human IgG1 Abs achieved by the expression of an Ig mini-repertoire composed of the secreted form of IgH-γ1, IgL-κ and IgL-λ chains. This human Ab mini-repertoire results from the combinatorial diversity generated by the rearrangement of 5 VH, 5 DH, 6 JH, 2 Vκ and 5 Jκ and 2 Vλ and 1 Jλ human gene segments during the natural development of mouse B lymphocytes. The unassembled human IgH and IgL tg genes rearrange during B cell development, along with endogenous mouse Ig genes. Since the human Ig transgenes encode only for the secretory form of Abs, expression of human Ig genes does not cause allelic exclusion (15, 16) of mouse Ig genes and hence the tg mice can mount an unrestricted immune response to human mAbs modifications. We applied the model to study the possible immunogenic potential of soluble Ab species of different sizes (monomers, dimers and oligomers) as generated by three different stress conditions. In addition, the tg mice were shown to be immune tolerant to a broad range of human IgG1s, demonstrating their applicability for the invesigation of the immunogenicity of a wide range of human therapeutic Ab preparations.

MATERIALS AND METHODS

Generation of Human IgG tg Mice

The three transgenes encoding for human IgH-γ1, Igκ and Igλ were generated as follows: IGH transgene – ET-cloning using BACs was performed to obtain 7 fragments (1.2 kb fragment containing IGHV4-59 element flanked by ~ 400 bp of endogenous sequence; 1.4 kb fragment containing IGHV3-30 element flanked by ~ 400 bp of endogenous sequence; 1.3 kb fragment containing IGHV3-23 element flanked by ~ 400 bp of endogenous sequence; 2.2 kb fragment containing IGHD3-3 and IGHD4-4 elements flanked by ~ 500 bp of endogenous sequence used as spacing region; 3.5 kb fragment containing IGHD2-8, IGHD3-9 and IGHD3-10 elements flanked by ~ 400 bp of endogenous sequence used as spacing region; 10 kb fragment containing IGHJ1 to IGHJ6 elements, intronic enhancer and switch (Sμ) region flanked by endogenous sequences used as spacing regions; 2.1 kb fragment containing IGHG1 exons 1 to 4 encoding the secreted IgG1 isoform, flanked by endogenous sequences used as spacing region) assembled together with 2 synthetic genes (1.3 kb fragment containing IGHV1-69 element flanked by ~ 400 bp of endogenous sequence; 1.2 kb fragment containing IGHV1-18 element flanked by ~ 400 bp of endogenous sequence) using exogenous restriction sites inserted during ET-cloning or gene synthesis. The human IGH tg sequence was excised from the vector backbone using an AscI/PmeI or AscI/NotI double restriction. Igκ transgene - ET-cloning using human or mouse BACs was performed to obtain 4 fragments (1.4 kb fragment containing IGKV3-20 element flanked by ~ 400 bp of endogenous sequence; 1.3 kb fragment containing IGKV1-17 element flanked by ~ 400 bp of endogenous sequence; 6.5 kb fragment containing IGKJ cluster, intronic enhancer and C region flanked by ~ 1 kb of endogenous sequence used as spacing region; 1.8 kb fragment containing the mouse Igk 3′ enhancer flanked by ~ 500 bp of endogenous sequence used

as spacing region) which were assembled together using exogenous restriction sites inserted during ET-cloning. The human Igκ tg sequence was excised from the vector backbone using an AscI/FseI double restriction. Igλ transgene - ET-cloning using human or mouse BACs was performed to obtain 4 fragments (1.3 kb fragment containing IGLV3-19 element flanked by ~ 400 bp of endogenous sequence; 1.3 kb fragment containing IGLV2-14 element flanked by ~ 400 bp of endogenous sequence; 3.8 kb fragment containing IGL J1-C1 region flanked by ~ 1 kb of endogenous sequence used as spacing region; 1.8 kb fragment containing the mouse Igk 3′enhancer flanked by ~ 500 bp of endogenous sequence used as spacing region) which were assembled together using exogenous restriction sites inserted during ET-cloning. The human Igκ tg sequence was excised from the vector backbone using an AscI/FseI double restriction.

The three transgenes were microinjected into the pronuclei of fertilized oocytes from C57BL/6 mice. Transgenic founders were mated with C57BL/6 mice and were maintained on this genetic background.

Human IgG1 Aggregates

An IgG1 monoclonal antibody (designated huMab-1) was chosen as a model antibody for the present study. Dimer and oligomer species were generated, isolated and characterized according to the methods described in (17). After applying the respective stress conditions (for the isolation of process-stressed material 6 g antibody bulk solution obtained from a large scale manufacturing campaign after the virus inactivation step in a buffer consisting of 90 mM Acetic acid, 110 mM Tris, and 20 mM Citric acid, pH 5.5, was used) the concentrated fractions were purified using a Superdex 200 preparative SEC column with 320 ml column volume and 600 mg sample load capacity at a flow rate of 2.5 ml/min. Sodium phosphate buffer (51 mM, pH 6.2) was used as elution buffer. The final monomer, dimer and oligomers fractions in phosphate buffer were sterile filtered and frozen in aliquots at −80°C. The isolated monomer fraction out of the process stress bulk was used as monomeric antibody. The protein content in the fractions was determined by measuring the absorption at 280 nm using a UV–VIS spectrophotometer Lambda 35 (Perkin Elmer). The absorbance at 320 nm, where protein absorption is minimal, is subtracted from the A280 values to correct for light scattering and for slight baseline offsets resulting from minor variations in cuvette alignment.

For liquid-chromatography/mass spectrometry (LC/MS) analysis the following method was used.

Analysis of chemical modifications of the species present in the isolated fractions was performed by LC/MS analysis of tryptic peptide maps. Prior to digestion, samples were denatured in 0.4 M Tris buffer, pH 7.5, containing 8 M Guanidine-hydrochloride. Trypsin digestion was performed by incubation at 37°C for 20 h in a digestion buffer (0.1 M TRIS/HCl, 1 mM $CaCl_2$, pH 7.5) containing trypsin (modified by reductive methylation) in a trypsin to mAb ratio of 1/20 (w/w) of the protein. Digestion was stopped by adding a 1% TFA solution. For chromatographic separation a Capillary-LC system (Agilent 1100 Series Capillary LC System) was used with a RP-C18 Phenomenex Jupiter column (250 × 0.5 mm, 5 μm, 300 Å) with a gradient of water / 0.1% formic acid to acetonitrile / 0.1% formic acid at a flow rate of 20 μl/min. Analysis of species eluting from the column was done by electrospray time-of-flight mass spectrometry (ESI-TOF-MS) in the positive ion mode on a Waters Q-Tof Ultima, achieving sequence coverage of greater than 95%. Each sample was analysed in duplicate with independent sample preparation.

Light Obscuration Measurements

A HIAC ROYCO instrument model 9703 (Skan AG, Basel, Switzerland) was used for LO measurements. A small volume method using a rinsing volume of 0.4 mL and 3 runs of 0.4 mL each was applied. Flow rate was set to 10 mL/min. First run was discarded and the average ± standard deviation of the last 2 runs was reported for each sample. Blank measurements were performed at the beginning of the measurements using particle-free water and PBS. Acceptance criterion for blanks was: "less than 5 particles >1 μm". System suitability test consisting of the measurement of count standards of 5 and 10 μm (Thermo Fisher count standards) with acceptance limits of ±10% the reported concentration for particles bigger than 3.0 and 5 μm, respectively, was performed in the beginning of measurement.

Mass calculation was done according to Barnard *et al.* assuming spherical particle shape and that each particle contained (by volume) 75% of protein and 25% of water with a protein density value of 1.43 g/mL: Estimated protein mass per size bin = (0.75) × (Volume) × (1.43 g/mL) × (Number of particles).

Resonant Mass Measurements (Archimedes)

The Resonant Mass Measurements (RMM) were performed using the Archimedes system (Model 1.8) from Affinity Biosensors, LLC (Santa Barbara, CA). Micro sensor chips with internal microchannel dimensions of 8 μm × 8 μm were used for all the experiments. The calibration of the sensor was done using 1 μm Duke polystyrene size standards (Waltham, Massachusetts, USA) diluted in water to approximately 106 particles per mL. The calibration was finalized after 500 particles were detected as recommended by the manufacturer. The particle density for proteinaceous particles was set to 1.32 g/mL according to the recommendation of the manufacturer. Measurements were performed in triplicates and the sensor

was filled with fresh sample during 40 s before each measurement. The limit of detection was automatically set by the software to 0.022 Hz. Each measurement stopped when a total of 100 particles were detected (corresponding to 10 min of measurement for process-related stress, 24 min for pH-stress and 33 min for UV-stress generated particles). The data was collected and analyzed using software ParticleLab version 1.8.570 (Affinity Biosensors, USA).

Mice and Immunization

Human IgG tg mice on C57BL/6 background used was heterozygous for human Ig. Wild-type C57BL/6 littermates were used as controls. All mice were kept in an animal facility at F. Hoffmann La-Roche, Basel. Experiments were conducted in accordance with protocols approved by the Veterinary Office of Kanton Basel.

Human IgG1 and its aggregated variants (all dissolved in 51 mM Sodium Phosphate buffer, pH 6.2), were administered s.c. twice a week for a total of seven injections (10 μg per injection) into either side of the abdomen. The antigen (Ag) was diluted in PBS to a final administration volume of 100 μl (2 × 50 μl). 50 μg of keyhole limpet hemocyanin (KLH) (Thermo Scientific) adsorbed in alum adjuvant (10 mg/ml Al(OH)3; Alhydrogel; Brenntag Biosector) was given s.c. once. Specifically, alum was added to the KLH preparation. The solution was then mixed and kept for 30 min at room temperature prior injection. Each mouse received 200 μl of KLH + alum solution (50 μg KLH + 1 mg alum).

Library Preparation and Sequencing

High molecular weight RNA (>200 bp) was extracted from mouse spleen, thymus, and bone marrow using the RNeasy Mini kit (Qiagen) including genomic DNA removal using the RNase Free DNase Set (Qiagen).

cDNA synthesis was performed using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). One μg of total RNA was reverse transcribed with RT Enzyme Mix (SuperScript™ III and RNaseOUT™), 1.25 μM oligo(dT)20, 1.25 ng/μl random hexamers, and dNTPs in RT Reaction Mix with 5 mM $MgCl_2$ in a total reaction volume of 20 μl at 25°C for 10 min and then at 50°C for 30 min. The reaction was terminated by incubating at 85°C for 5 min and chilled on ice. 0.1 U/μl E.coli RNaseH (Invitrogen) was added and the reaction incubated at 37°C for 20 min. cDNA was stored at −20°C.

Primers (Microsynth) were designed to target the human Ig heavy and light chain sequences as follows: VH1-18 5′-ATGG TATCAGCTGGGTGCGAC-3′, VH1-69 5′-AGGCACCT TCAGCAGCTATGC-3′, VH3-23/30 5′-GCAGACTCCG TGAAGGGCCG-3′, VH4-59 5′-AAGGGACTGGAGTG GATTGGG-3′, Cγ1-PCR 5′-AGTAGTCCTTGACCAG GCAGC-3′, Vκ1-17 5′-GCCAGGTGTGACATCCAGAT G-3′, Vκ3-20 5′-AGGCACCCTGTCTTTGTCTCC-3′, Cκ-PCR 5′-CAGATGGTGCAGCCACAGTTC-3′, Vλ3-19 5′-GGGACAGACAGTCAGGATCAC-3′, Vλ2-14 5′-TGCACTGGAACCAGCAGTGAC-3′, Cλ-PCR 5′-ACAGAGTGACAGTGGGGTTGG-3′. Fifty ng of cDNA was amplified with 0.2 μM primers, 25 μM dNTP, and 0.04 U/μl Fast Start High Fidelity Polymerase (Roche) in High Fidelity Buffer 2 (with $MgCl_2$) (Roche) in a total volume of 15 μl at 95°C for 5 min, 40 cycles of 95°C for 45 s, 63°C for 45 s, and 72°C for 60 s, and a final elongation at 72°C for 5 min. Amplification products were pooled per tissue per animal before sequencing library preparation.

Sequencing libraries were prepared from 250 ng of pooled PCR products using the Illumina TruSeq DNA Sample Preparation kit version 2 (Illumina) following the manufacturer's instructions. Sequencing libraries were quantified using the Kapa Library Quantification kit (Kapa Biosystems) and quality controlled by capillary electrophoresis on a Bioanalyzer using DNA1000 chips (Agilent Technologies).

Libraries were sequenced on a MiSeq sequencer (Illumina) for 2 × 250 cycles using version 2 MiSeq reagents (Illumina). Ten percent of PhiX control library (Illumina) was spiked into the MiSeq lane as a sequencing control and to increase the sequence complexity in the run.

ELISA

For determination of ADAs, ELISA plates (Nunc Immuno MaxiSorp, Rochester, NY) were coated overnight with hIgG1 and its aggregated variants (5 μg/ml) in coating buffer (0.1 M NaHCO, pH 9.6). Prior to use, the plates were washed three times with PBS containing 0.05% (*w/v*) of Tween-20, followed by 1 h blocking with PBS containing 1% (*w/v*) BSA. The serum samples serially diluted (starting dilution 1:50) in serum dilution buffer (PBS + 1% BSA + 1% FCS) were incubated for 2 h at RT. Subsequent to washing, binding ADAs were detected using alkaline phosphatase-conjugated goat anti-mouse IgG (Fc gamma specific), Jackson Immuno Research. For quantification of human IgG1 heavy chain, plates were coated with mouse anti-human IgG (heavy chain specific) and detected with biotin mouse anti-human IgG1 followed by alkaline phosphatase-conjugated streptavidin. For determination of human Igκ, plates were coated with mouse anti-human κ and detected with rat anti-mouse IgG1; human Igλ was determined by coating the plate with mouse anti-human λ and detected with biotin rat anti-mouse λ1, λ2 and λ3. ADAs IgG titers in serum were measured by endpoint titer calculating the OD (405 nm) cutoff as the average of the OD of the naïve sample multiplied by ~ 3-fold the SD of the OD of the naive sample. KLH IgG titers are indicated as dilutions reaching half-maximal absorbance at 405 nm. . Antibodies

were purchased from BD Biosciences, unless otherwise specified.

Flow Cytometry

For detection of human IgG1 secreting cells, BM cell suspensions were stained with FITC-conjugated anti-mouse IgM, Jackson Immuno Research, anti-B220 (Pe-Cy7), anti-CD45 (APC-Cy7), mixture of PerCP-Cy5.5-anti-CD11b, anti-CD11c and anti-Gr-1, (to exclude myeloid cells), biotin-labelled anti-CD138 followed by streptavidin-PE followed. After permeabilization, cells were incubated with mouse APC-anti-human IgG (heavy chain specific).

Splenic follicular and MZ B cells were identified by staining with anti-B220 (Pe-Cy7), anti-CD23 (PE) and anti-CD21 (PerCP-Cy5.5). For identification of Pro/pre, newly formed and re-circulating B cells, BM cell suspensions were stained with FITC-conjugated anti-mouse IgM and anti-B220 (Pe-Cy7). Thymocytes were stained with anti-CD4 (PE) and anti-CD8 (PerCP).

In all cases Fc-receptors were blocked with rat IgG2b anti-mouse CD16/32 (1/100; 2.4G2). Antibodies were purchased from BD Biosciences, unless otherwise specified. Data were acquired on FACSCanto (BD) and analyzed with FlowJo software (TreeStar).

IVIG1 Purification and its Fragmentation

Human IVIG-1 was purified from human IVIG (Jackson Immuno Research) using a Camelid single domain Ab (CaptureSelect) specific to human IgG-CH1. For purity assessment, the eluate was coated on an ELISA plate and detected with biotin anti-human IgG1, IgG2, IgG3 (Sigma-Aldrich) and IgG4 followed by alkaline phosphatase-conjugated streptavidin. Antibodies were purchased from BD Biosciences, unless otherwise specified.

The purified IVIG-1 fraction was digested with papain in a digestion buffer (0.1 M Tris–HCl pH 7.4, 0.04 M EDTA) containing 0.4 mg/ml IgG1, 0.08 mg/ml papain and 10 mM cysteine for 3 h at 37°C. The sample was loaded into an ÄKTA FPLC (GE Healthcare) on a 1 ml column with AF_ProteinA-650 F (Tosoh Bioscience) affinity resin with a flow rate of 0.1 ml/min. After washing with buffer containing 50 mM Na_2HPO_4/NaH_2PO_4 pH 6.0, bound protein was eluted with 50 mM citrate buffer pH 3.01. Flow through and eluate were collected, concentrated with a Vivaspin 2 Centrifugal Concentrator (Vivaproducts Inc.) with a 30 kDa cut-off to 100 µl and loaded to a Superdex S200 10/300GL (GE Healthcare) column equilibrated with PBS buffer and a flow rate of 0.5 ml/min for gel filtration. Fractions were collected, concentrated as before and analyzed via HPLC with a C8 poroshell 5u, 1 × 75 mm microbore column (Agilent) at 70°C and a flow rate of 0.68 ml/min.

Depletion of CD4 T Cells

$CD4^+$ T cells were depleted with an anti-CD4 mAb (clone GK1.5) given by four i.p. administrations of 500 µg Ab (4 days before and 3, 10 and 17 days after the first Ab aggregate administration). Depletion of $CD4^+$ cells was determined by flow cytometry using an anti-CD4 mAb, clone RM4-4, which is known to bind an epitope distinct from the one recognized by the GK1.5 mAb. As indicated in Fig. S4, this protocol led to ~ 95% depletion.

Statistical Analysis

For assessing statistical significance of the observed changes, we used one out of three tests: Student's t-test, Welch's t-test or Wilcoxon signed rank test (JMP 10, SAS). Note that this ordering is linked to statistical power and thus indicates preference of application, but in all cases distributional properties of the data have been checked carefully to ensure usage of the appropriate test. In cases where only upper limits of the titers could be obtained for at least one treatment group, we applied the non-parametric Wilcoxon test which results in very conservative significance statements, even when larger changes are evident from the data. P values lower than 0.05 were indicative of significance and labeled with one asterisk. Two and three asterisks indicated P values lower that 0.01 and 0.001, respectively.

RESULTS

Expression of Human IgG1 in Transgenic Mice

Transgenic mice were generated by pronuclear microinjection of a mixture of the three tg constructs described in Fig. 1a. In these constructs germline V gene segments were arranged such as to permit normal rearrangement of the selected 5 V_H, 5 D_H and the 6 J_H gene elements and their expression as IgH-γ1 chain; of 2 Vκ and the 5 Jκ gene elements for the IgL-κ chain; and 2 Vλ and 1 Jλ gene elements for the IgL-λ chain. The V genes used in these constructs were chosen based on their predominant usage in human peripheral blood (18), as well as in tg mice bearing the human IgH V region (19). The rearrangement of human Ig transgenes could be readily detected by specific PCR amplification using genomic DNA from peripheral blood cell preparations of tg mice (not shown). In addtion, we analyzed the repertoire of expressed human Ig sequences by deep sequencing of mRNA isolated from BM, spleen and thymus from mice of tg lines 17

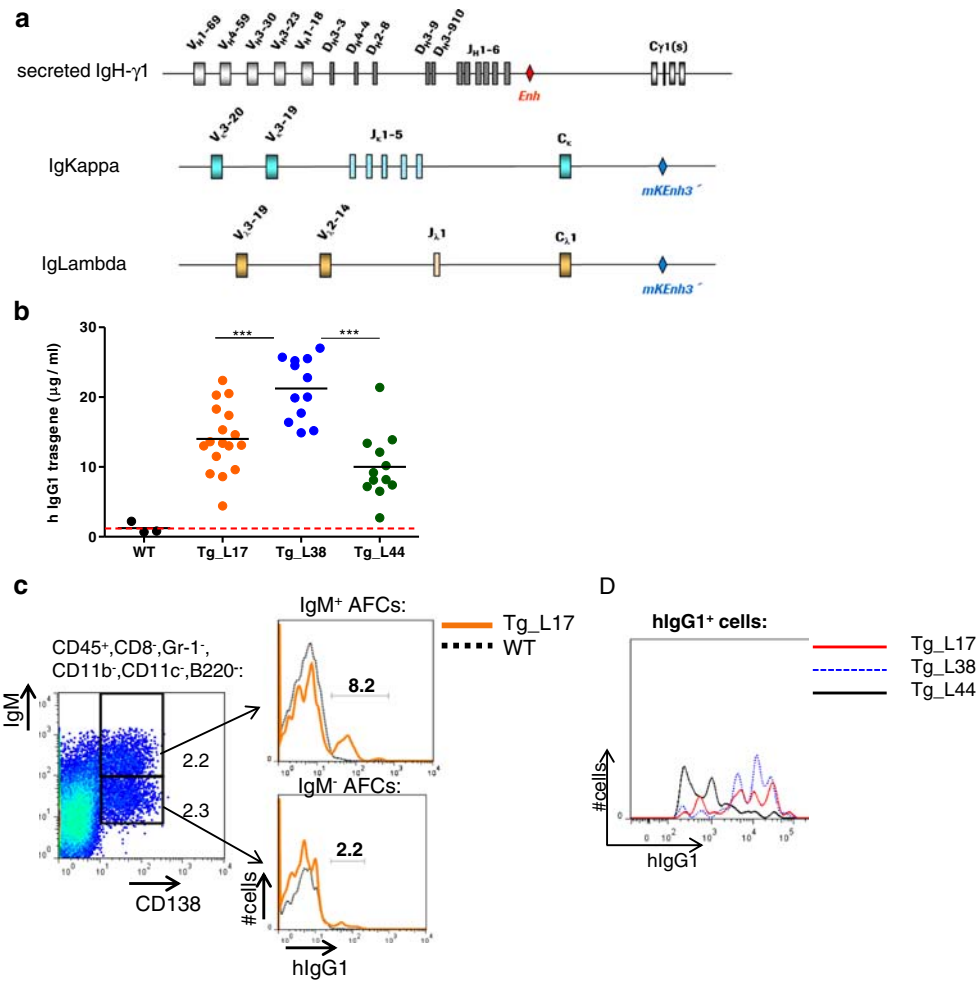

Fig. 1 hIgG transgene constructs and their protein expression. (**a**) The IgH-γ1 tg is composed of 5 V regions, 5 D elements and 6 J families as well as enhancer and exons encoding the soluble form of constant γ1. The Igκ tg is composed of 2 V families and J elements as well as constant κ. Yet, the Igλ tg is composed only by 2 V families, 1 J element, the constant λ and enhancer. (**b**) hIgG1 concentrations in serum of WT and Tg mice. Data shown represent mean ± SEM of 10–12 mice/group. $^{**}P < 0.01$ and $^{***}P < 0.001$. (**c**) hIgG1 expression by IgM^+ (upper plot) and IgM^- (lower plot) $B220^{dull}$ BM cells in WT and Tg_L17 mice. (**d**) Histogram analysis of hIgG1 expression level among the three Tg lines. Representative plots representative of three independent experiments are shown.

and 38. An array of the most frequent sequences found is presented in Fig. S1. The sequence data demonstrate that all human V genes rearrange and generate coding joints making use of the D and J elements included in the tg constructs to similar frequencies in the two tg lines. The Fig. S1 also shows that rearrangements of both the IgH and IgL genes display N sequences leading to non-germline encoded aminoacids at the joining sites (marked in red). Altogether we detected an average of 4.3, 1.5 and 1 aminoacids per rearrangement as contributed by N-sequences in V_H, Vκ and Vλ gene rearrangements, respectively. The analyzed sequences also revealed 20, 5 and 5 different aminoacid exchanges due to somatic mutations in 91 V_H, 13 Vκ and 20 Vλ different gene rearrangements, respectively (Fig. S1).

Of 11 tg mouse lines generated, nine had integrated the three tg DNA constructs (data not shown) and of these, only three lines (17, 20, 21) expressed all three human Ig genes (data not shown). The tg lines 17, 38 and 44 were thus chosen for further experimentation and are henceforth referred to as Tg_L17, Tg_L38 and Tg_L44. The levels of human IgH-γ1 chains in serum of these tg mice has been demonstrated by ELISA (Fig. 1b) and has been estimated as 10 µg/ml ± 1.4 (Tg_L44), 14 µg/ml ± 1.1 (Tg_L17) and 21 µg/ml ± 1.3 (Tg_L38), although values for individual mice in each line varied widely (Fig. 1b). Moreover, human κ and λ L chains could not be detected associated with human IgH-γ1 chain by ELISA (Fig. S2A and B). Instead, human κ was detected only in association with mouse IgH chains (Fig. S2C), and human λ could only be detected associated with mouse IgG bearing λ light chain (Fig. S2D). Clearly, the amounts of human H and L chains produced in tg mice do not correlate, since the highest levels of human L chains were found in Tg_L44 mice, despite having a lower content of human H chains (Fig. 1b and S2).

Collectively, these data indicate that the low levels of human Ig proteins produced in tg mice are diluted by mouse Ig molecules such that pure human IgG1 molecules are not detectable.

We next investigated the cellular sources of human Ig proteins in the tg mice. As only B and T cells express recombination-activating gene 1 (RAG1) and 2 (RAG2), the proteins responsible for rearrangement of lymphocyte antigen

receptor genes, we analyzed human IgG1 expression in these two cell subsets. FCM analysis revealed that surface IgM-positive, B220-negative plasmablasts in the bone marrow (BM) were the major source of intracellular human IgG1 followed by B220-negative, IgM-negative, probably IgG^+, plasmablasts (Fig. 1c). Interestingly, the intensity of intracellular human IgG1 roughly correlated with the different levels of secreted human IgG1 detected by ELISA (Fig. 1b) for the three tg lines: Tg_L38 > Tg_L17 > Tg_L44 (Fig. 1d). In contrast, expression of human IgG1 was absent in mature splenic B cells, in thymocytes and in BM immature pre-B cells (data not shown). Since the tg human IgH genes encode only the soluble form of IgG1 we conclude that transgenic Ig proteins are mainly synthesized and secreted as passenger molecules in mouse bone marrow plasma cells.

Analysis of immune organs of the tg mice did not reveal any abnormalities in Tg_L17 and Tg_L38. FACS analysis of cell subsets in the BM, spleen and thymus of these mice revealed a normal distribution of pre-B and mature B cells in BM, of follicular and marginal zone B cells in the spleen and of thymocytes and mature T cells in the thymus (Fig. S3). In contrast, the Tg_L44 line revealed strong depletion of immature B cells in the BM and mature B cells in spleen and a relative accumulation of splenic marginal zone B cells ($CD21^{high}$/$CD23^{low}$) compared to follicular B cells ($CD21^{low}$/$CD23^{high}$) (Fig. S3A and B). Since an uncompromised immune system is essential for the assessment of the immune response to human Abs in our tg mice, we excluded Tg_L44 from further studies.

Immune Responses in Human IgG Transgenic Mice

The immune competence of human IgG tg mice was first assessed by s.c. immunization with a single dose of 50 µg KLH in alum. Since the KLH-specific Ab response in tg mice was comparable to wt mice, we concluded that the expression of human IgG genes did not compromise their immune capacity (Fig. 2a). Next, we assessed the tolerance status of the tg mice towards a human IgG1 model Ab (designated as "huMAb-1"). In contrast to the Ab response observed in wt non-transgenic littermate control mice, human IgG tg mice were clearly unresponsive to huMab-1 (Fig. 2b). But, since therapeutic Abs are administered to patients without adjuvants and in repeated doses, the tolerance status of the tg mice needed to be confirmed with a comparable immunization

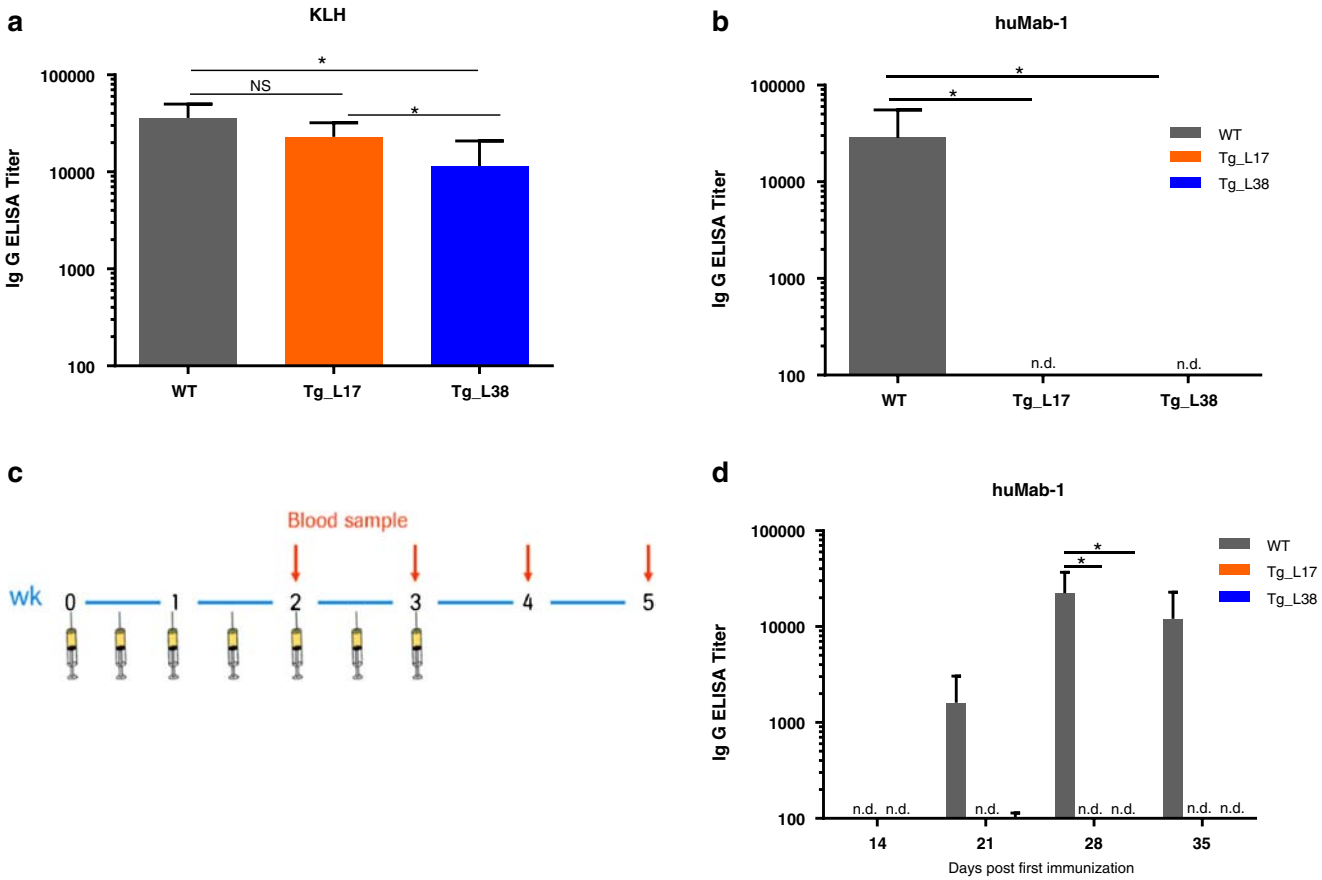

Fig. 2 hIgG transgenic mice mount an immune response to foreign antigen but are tolerant to native monomeric huMAb-1. The tg mice and wt controls were immunized s.c. with 50 μg of KLH (**a**) or the model huMAb-1 (**b**) adsorbed in alum and 20 days later, specific IgG levels were measured in serum by ELISA. (**c**) Schematic outline of the experiment without adjuvant**.** (**d**) ELISA of hIgG1 specific mouse IgG response at indicated time points. Mean IgG ELISA titersn + SEM ($n = 5$) are shown; n.d., not detectable. *$P < 0.05$ and ns, not significant.

scheme. Hence, we used an immunization protocol using repeated s.c. aplications (10 μg per injection; twice a week; for a total of 7 injections) in the absence of adjuvant (Fig. 2c).

As shown in Fig. 2d, mice from tg lines Tg_L17 and Tg_L38 were immune tolerant to huMab-1 also upon the repeated immunization regimen depicted in Fig. 2c. Given the high degree of diversity of human variable region (V) gene elements, we next determined whether a broad range of V sequences of human Abs can be tolerated by our tg mice. To study this question we used purified polyclonal human IgG1 from commercial IVIG preparations (96% pure IgG1, henceforth referred to as IVIG-1). Immunization with this IVIG-1 preparation elicited a strong Ab response in wt mice while the response was largely abrogated in tg mice (Fig. 3a). Because the tg human IgH "minirepertoire" is built up from only 5 human V_H, 2 Vκ and 2 Vλ gene segments, we hypothesized that the weak Ab response still detectable in tg mice was mainly composed of anti-idiotypic Abs. Using purified fragments we found that the anti-IVIG-1 Abs elicited in tg mice failed to bind the purified Fc fragment of IVIG-1 (Fig. 3b) while weakly binding the enriched polyclonal Fab fragments from IVIG-1 (Fig. 3c). In contrast, Abs from wt mice were mainly directed against the Fc part and only a minor response was against the Fab part of IVIG-1, confirming that Fc is the immunodominant part of Igs (22). We concluded that the weak Ab response elicited in Tg_L17 mice by polyclonal IVIG-1 was mainly directed towards idiotypic V region or undefined conformational determinants.

Antibody Aggregates did not Break Tolerance in Human IgG Transgenic Mice Unless Significantly Covalently Modified

Having established the immune tolerance status of human IgG tg mice towards human IgG1 Abs, we next studied their capacity to assess potentially immunogenic modifications of Ab preparations.

For these experiments we used soluble aggregated forms of huMab-1 generated by different stress conditions, as described in (17). These included (1) process-related stress, i.e. impurities as separated from manufacturing processes before further purification, (2) exposure to low pH (1 h, pH 2.5) and (3) exposure to artificial light (UV/Visible light, 20 h 765 W/m^2) which represents significant artificial light stress.

After removal of precipitates by 0.2 μm pore size filters, the resulting monomeric, dimeric and larger oligomeric species were isolated as described in (17) by preparative ion exchange (IE) and subsequent size exclusion chromatography (SEC). The enriched fractions were characterized by biochemical, structural and functional methods and were shown to differ in their physical and biological properties depending on the type of stress applied (17). It is important to note, that the highly aggregated oligomeric fractions showed very low residual content of subvisible particles measured by Light Obscuration and Resonance Mass Measurement (see Supplementary Tables 1 and 2 for further details), thus excluding the possibility of contribution of subvisible particles (in either the micrometer- or sub-micrometer- size ranges) to the effects observed. Significant chemical modifications of the primary structure (i.e. broad oxidation of methionine and tryptophan residues) were only observed in fractions derived from material after extensive exposure to artificial light. It was also shown by transmission electron microscopy (TEM) that Ab dimers resulting from different stress conditions had significant conformational differences (17).

The immunogenic properties of these enriched huMab-1 aggregates were compared with native huMab-1 by immunizing tg and wt mice using the protocol described in Fig. 2c. Immunization with enriched dimer fractions from huMab-1 subjected to the three types of stress conditions revealed that whereas all three types of huMab-1 dimers were immunogenic in wt animals, only light induced aggregates including dimers with covalent modifications elicited an immune response in tg mice (Fig. 4a-c). Similarly to dimers, huMAb-1 oligomers formed upon exposure to the stress conditions of low pH and process-related stress also were not

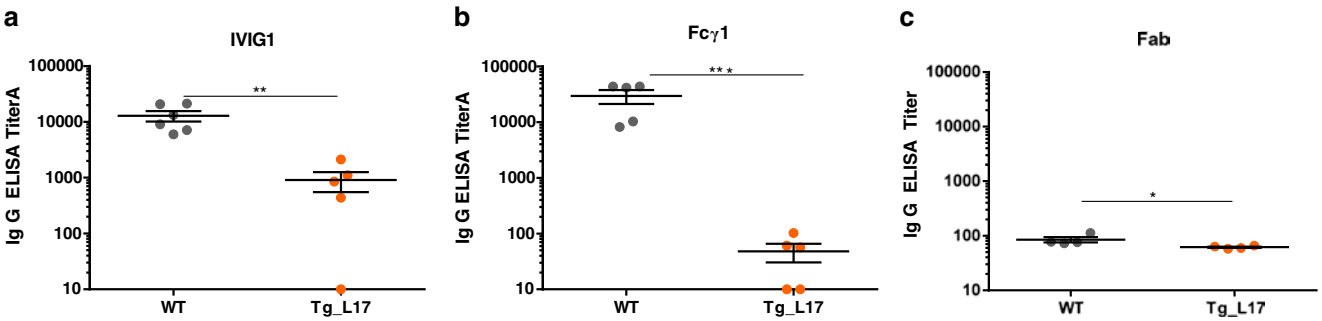

Fig. 3 hIgG transgenic mice are tolerant to a broad specificity of human IVIG1. WT and Tg_L17 were immunized with IVIG1 as indicated in Fig. 2a. IVIG1 (**a**), Fcγ1 (**b**) and polyclonal Fab (**c**) specific IgG response were determined by ELISA 28 post the first immunization. Data shown represent mean IgG ELISA titers ± SEM of 5 mice/group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ns, not significant.

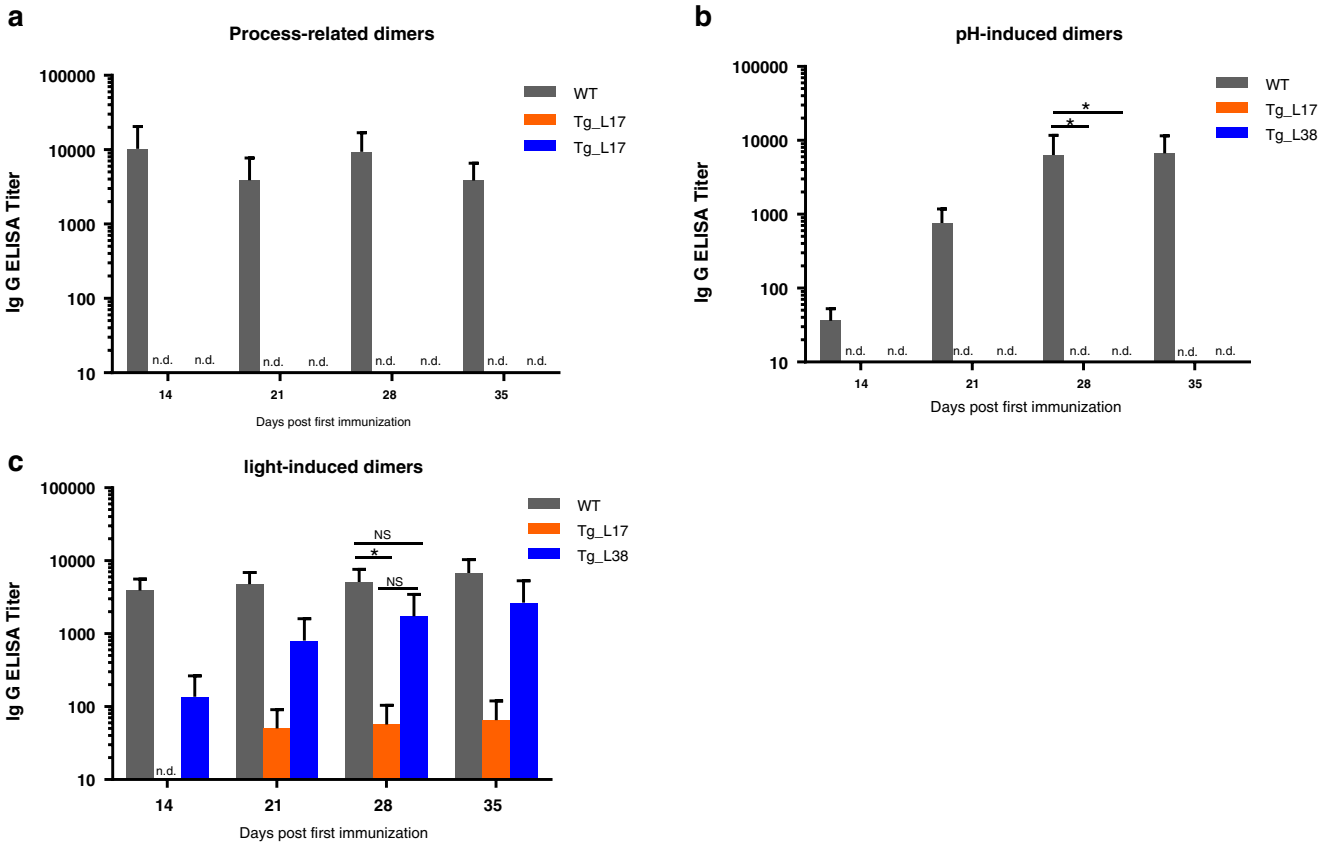

Fig. 4 Immune response induced by stressed dimers. WT and the two tg lines were immunized with process-related (**a**), pH (**b**) and light (**c**) stressed huMab-1 dimers as indicated in Fig. 2c and specific IgG responses were determined by ELISA at indicated time points. Mean IgG ELISA titers + SEM ($n = 5$) are shown; n.d., not detectable. *$P < 0.05$ and ns, not significant.

immunogenic in tg mice (Fig. 5a and b). In contrast, soluble oligomeric species also containing covalent modifications generated by light-stress elicited a strong Ab response both in wt and tg mice (Fig. 5c). Interestingly, light-stressed huMab-1 monomers failed to evoke an immune response in tg mice (Fig. 5d).

In addition, we found that Abs present in the serum of tg mice immunized with light-stressed huMab-1 oligomers also bind to light-stressed huMab-1 monomers (Fig. 5e) thus indicating that the Ab response is not only directed to conformational epitopes present in the aggregates but also to chemical modifications possibly representing neo epitopes present in the light -stressed huMab-1 monomers, as well. In addition, we found that ADAs raised against light-stress induced huMab-1 oligomers also bind to unmodified, native huMab-1 (Fig. 5f). This fraction of anti-huMab-1 Abs can only be directed to native epitopes and is hence reminiscent of self-reactive B cell clones specific for human IgG1 that persist in hIgG1-tolerant transgenic mice.

In sum, soluble dimers and oligomers of huMab-1 induced by significant light-exposure could readily break tolerance and induce strong immune responses in tg mice, while monomers treated with the same conditions showed no significant immunogenicity in tg mice (Figs. 4c, 5c and d). Additionally, also aggregates with or without covalent modifications as significantly stressed by pH or process, were not able to break tolerance in the tg mice (Fig. 5a and b).

Tolerance Breakdown depends on neo-epitopes

Light-stress induced soluble oligomers contain similar aggregate species with regard to size as determined by SEC as the non-immunogenic pH-stressed and process-related oligomers, with varying distribution of aggregate species between the stress conditions and a trend to larger aggregates for light stresses material (Table I). A search was therefore conducted for additional parameters which could explain their distinctive immunogenic potential. By LC/MS-analysis, succidinylation, deamination and methionine oxidations were detected in all preparations, including native huMab-1 monomers (Fig. 6a), representing common modifications also found in other Ab solutions. Notably, light-stress induced soluble oligomers showed substantial differences in the LC/MS peak pattern compared to the native monomeric huMab-1. Oxidation of methionine and tryptophan residues were amongst the main modifications (Fig. 6a). These amino acid modifications were also detected in similar abundance in the other fractions of

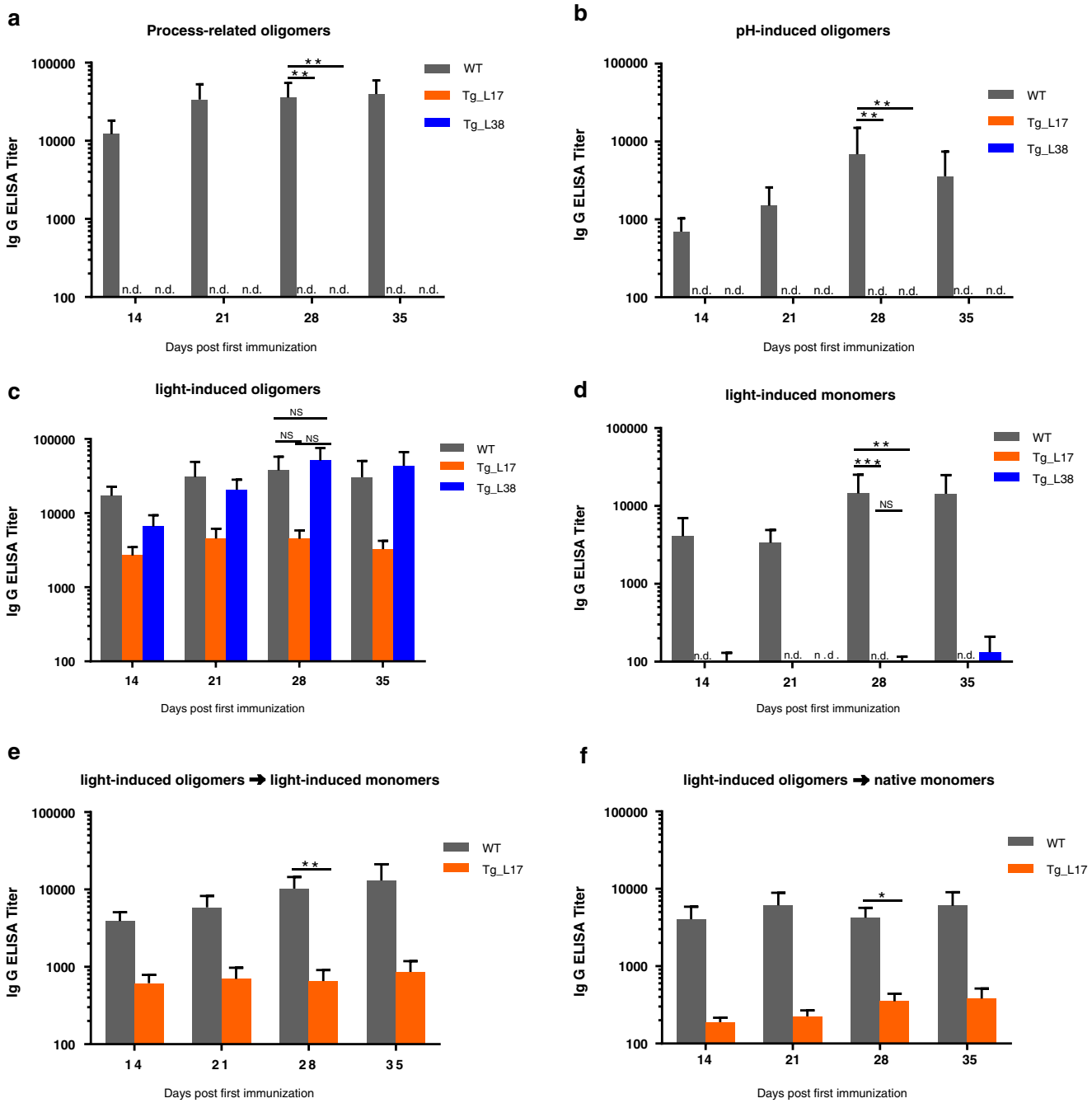

Fig. 5 Immune response induced by stressed oligomers and light-stressed monomers. WT and the two tg lines were immunized with process-related (**a**), pH (**b**) and light (**c**) stress induced huMAb-1 oligomers as well as light-stressed monomers (**d**) as indicated in Fig. 2a and specific IgG responses were determined by ELISA at indicated time points. IgG ELISA using serum from WT and Tg_L17 mice immunized with light-stress induced huMAb-1 oligomers as in *C* were performed to determine binding to light-stressed huMAb-1 monomers (**e**) and native monomer (**f**). Mean IgG ELISA + SEM ($n = 5$) are shown; n.d., not detectable. $^{**}P < 0.01$, $^{***}P < 0.001$ and ns, not significant.

light-stressed preparations, including dimers and monomers (Fig. 6c), but modifications were in very low abundance, or not detected in oligomers (Fig. 6a) or dimers (Fig. 6b) formed after pH or process related stress. Besides those previously stated, other modifications were detected in the light-stressed material which could not be further identified (i.e., appearing as "unknown peaks" in LC/MS-peptide map chromatograms) due to the compexity of possible interaction products, like di-tyrosin species or other more complex oxidation products (23).

Thus, the immunogenic light-stress induced huMab-1 aggregates display amino acid modifications which were not

Table I Composition of huMAb-1 Dimers and Oligomers Generated upon Light, pH and Process-Related Stress

Enriched Ab aggregate fractions	Monomer (%)	Dimer (%)	Trimer (%)	Tetramer-hexamer (%)	HMW aggregates (%)
UV-stress-induced dimers	23.4	63.7	8.2	3.8	0.5
UV-stress-induced oligomers	13	15.2	20	34.6	16.8
pH-induced dimers	4.4	86.7	8.5	0.4	–
pH-induced oligomers	4.9	15.4	43.4	23.8	12.6
process-related dimers	1.3	87.2	10.1	1.4	–
process-related oligomers	1.3	7	68.2	23.5	–

Area percentages determined by analytical SEC are given

present in the immune tolerated pH-stressed and process-related aggregates. It is therefore likely that the chemical amino acid modifications were rendering light induced oligomers more immunogenic.

Antibody Response to Light-Stressed Ab Oligomers Requires T Cell Help

Oligomeric aggregates, being large molecules ranging from 600 kDa up to 3 MDa, are conceivably suited for crosslinking of membrane Ig receptors on the surface of B cells resulting in a T cell-independent (TI) humoral immune response (24, 25). In order to understand whether light-stressed oligomers, behaved as TI or T-cell dependent (TD) Ag, we treated wt and tg mice with the GK1.5 CD4 depleting Ab (26) (Fig. S4) prior to immunization with light induced huMab-1 oligomers. As controls, non-CD4 depleted wt and tg mice were similarly immunized. Figure 7 shows that the immune response against light induced oligomers was totally abolished in tg and wt mice depleted of CD4 T cells.

DISCUSSION

ADA responses are of concern in the use of biologic therapeutic agents, since they may result in neutralization of the therapeutic protein, acceleration of drug clearance or even induce hypersensitivity reactions (5, 10), although in most cases, ADA responses do not significantly impact the clinical outcome. Several tg mouse models which are immune- tolerant to a specific human protein have been proposed as tools for the assessment of aggregate-induced immunogenicity (27–31). However, little has been published using human IgG tolerant mouse models to elucidate the attributes that render therapeutic mAbs immunogenic (13, 14). While the transgenic system applied by Filipe *et al.* lack the human IgG isotypes (13), the system by Bi *et al.* (14) allows the expression of a large repertoire of human IgG Abs. However, the last is hampered by the allelic exclusion of the murine Ab repertoire as mediated by the membrane form of the transgenic human Abs, thus forcing a laborious crossing of the transgenic line with knock-out Ig alleles and one wild-type allele. Here, we describe the generation of human IgG tg mice expressing a human Ig mini-repertoire composed of soluble human Ig-γ1 H and of the human Ig-κ and Ig-λ L chains. The use of only the secretory form of human IgG1 was chosen to avoid the restriction of the host murine B cell repertoire resulting from allelic exclusion as imposed by membrane bound transgenic Ig H proteins (15, 16) . Therefore, the two tg lines used in this study, Tg_L17 and Tg_L38, display an unaffected B cell compartment and express low levels of human Ig, which is nonetheless sufficient to promote and maintain immune tolerance to a broad range of human IgG1.

Numerous IgG tg mouse systems bearing complex transgenic constructs have been generated for the production of humanized therapeutic mAbs (32–36). These mice have been shown to efficiently produce high-affinity human mAbs (37–39). However, in all cases the tg mice also express surface human Ig receptors, which, firstly, will induce poor signaling when coupled to mouse co-receptor molecules Igα/Igβ impacting B cell maturation and secondly, result in allelic exclusion of mouse Ig leading to extensive B cell depletion and a restricted B cell repertoire (15, 16).

To overcome these issues, Bi and colleagues constructed a human Ig tg mouse bearing only heterozygous deletions of the mouse IgH and IgL loci in order to allow expression of both human and mouse Ig repertoires (14). In this system only 2.5% of B cells express human Igs thus permiting the formation of normal murine immune responses against immunogenic variants of human IgG. However, the breeding and maintainance required for three knockout (mouse IgH, Igκ and Igλ L) and two tg loci (human IgH and IgL) is laborious and impractical for routine screenings requiring large numbers of animals. In contrast, the tg mice described herein combine three tg constructs coding for IgH-γ1, IgL-κ and IgL-λ chains co-integrated at a single hemizygous locus of the mouse genome by co-injection into mouse oocytes and thus large numbers of animals can be easily generated.

The minirepertoire expressed in the tg mice is the result of combinational diversity generated by the rearrangement of 5 V_H, 5 D_H, 6 J_H; 2 Vκ, 5 Jκ; and 2 Vλ and 1 Jλ tg during B cell

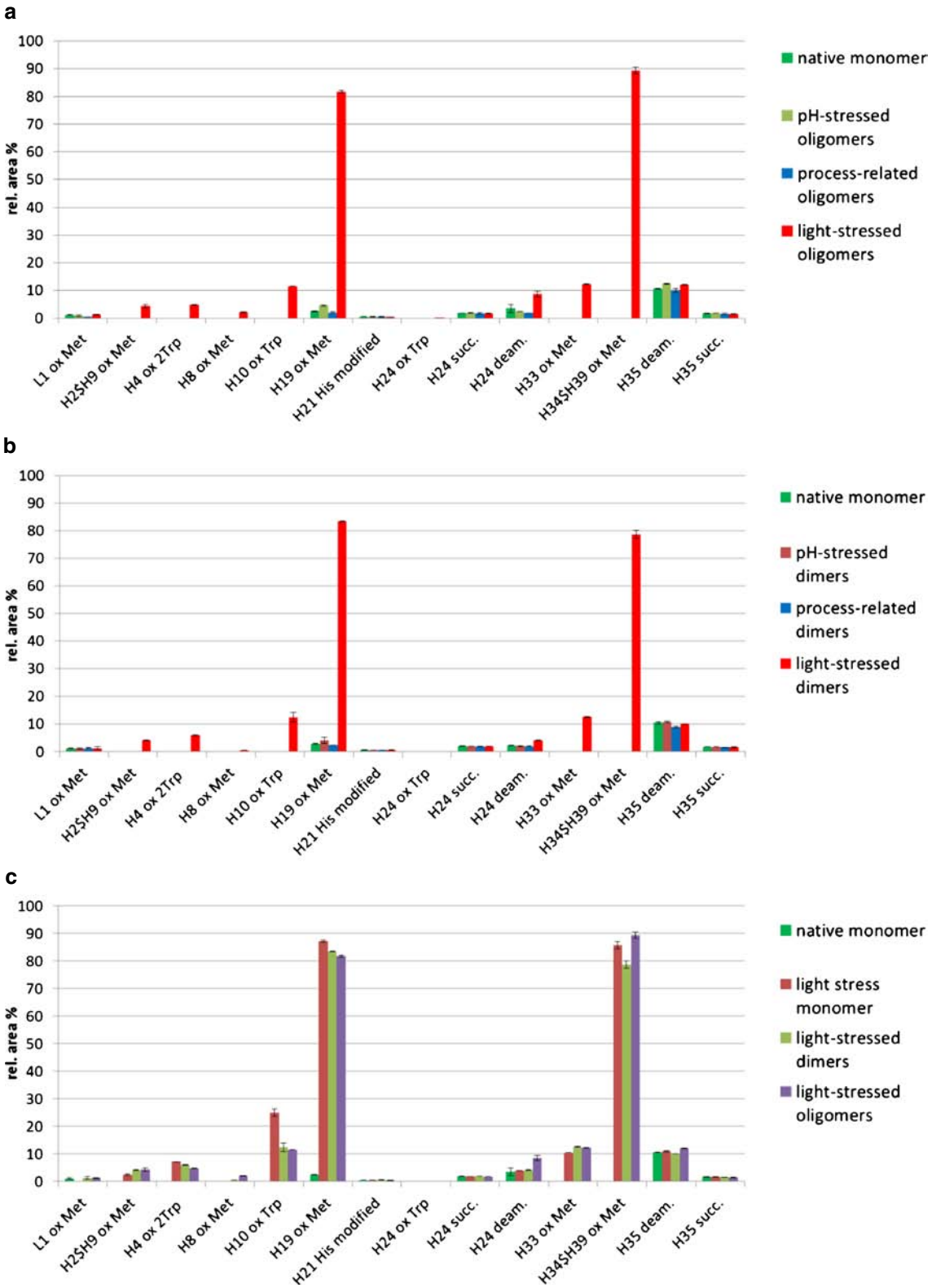

Fig. 6 Results of LC/MS characterization of native monomer and isolated fractions after light, pH and process-related stress. Tryptic digests of native huMAb-1 (A-C), huMAb-1 oligomers (**a**) and dimers (**b**), isolated light-stressed huMab-1 monomers, dimers and oligomers (**c**) were analyzed by LC/MS for the modifications indicated in the figure. The y-axis in displays the relative percentage of the area of the extracted masses from Total Ion Current (TIC) from the modified- relative to the corresponding unmodified peptides (area % modified + area % unmodified = 100%). The x-axis indicates the modifications analyzed in tryptic peptides of Light (L) or Heavy (H) chains. Ox Met, ox Trp: oxidation of methionine or tryptophan. Deam.: deamidation of histidine. Succ.: succinimide intermediate of asparagine. Disulfide bridges linking peptides are indicated by "$". The *bars* show average of two independent analysis, *error bars* indicate the min/max values.

development. All germline V gene segments of our tg constructs rearrange efficiently to their corresponding D and/or J elements, including the typical appendage of N sequences at the joining ends and the insertion of somatic mutations (Fig. S1). The presence of N sequences, while expected for IgH genes, is unusual for IgL genes (20). This finding indicates that the germline IgL tg genes undergo rearrangement during the pro-B-cell stage, when endogenous murine IgH genes rearrange and, like those, apend N nucleotides at their VL-JL joinings (20). In theory, the tg minirepertoire is far smaller than the normal human Ab repertoire (50 VH × 25 DH × 6 JH; 60 Vκ × 5 Jκ; 30 Vλ × 4 Jλ) (40), but in practical terms, the human Ig genes are not randomly used and some Ig-V genes are preferentially expressed (41, 42). Accordingly, we selected for the tg constructs Ig-V (and –D) genes among those most frequently represented in human peripheral blood B cells (18, 43, 44). In fact, we have observed that the tg mice responded only weakly upon immunization with polyclonal IVIG-1, as compared with wt mice, thus suggesting tolerance to most IgG1 proteins present in human serum. In wt animals the immune response was dominated by Abs against the Fc fragment of IVIG-1, while anti-Fab Abs represented only a small fraction of the total anti-IVIG-1 Ab response. We interpret the residual Ab response to human IVIG-1 in tg mice as the result of rare anti-idiotypic and possibly also anti-conformational epitope responses. We conclude that our tg mice, though expressing only a limited number of human Ig V genes, generate a large repertoire of human Ig V gene sequences and hence are unresponsive to a large array of human IgG1 Abs. This tg mouse model should therefore be suitable for the assessent of the immunogenic properties of a large array of different therapeutic Abs.

Furthermore, we have found that only soluble huMab-1 aggregates carrying a high degree of chemical modifications (neo-epitopes) within the primary structure were immunogenic in the tg mice. Importantly, under this condition, the induced ADAs also recognized native huMab-1, thus proving breakdown of tolerance to the self hIgG1. Soluble aggregates that did not contain these chemical modifications or did contain other chemical modifications were not immunogenic in tg mice at all. This finding is in agreement with similar observations made by others. Indeed, Joubert and colleagues found that only mAb aggregates containing specific chemical modifications induced secretion of innate cytokines in PBMC cultures (21). And recent studies in wild-type mice using a murine mAb have pointed to the strong immunogenic potential of large, insoluble Ab particulates and glass-adsorbed microparticles treated with UV light (45, 46). Furthermore, the immunogenicity of human IFNβ interacting with metal particles, although not specifically assessed but most likely due to oxidation of tryptophan residue, was demonstrated in human IFNβ tg mice (47). More recently, Bi and colleagues also showed in the tg mouse model aforementioned the immunogenicity of stirred aggregated mAb carrying methionine and tryptophan oxidation (14). Oxidized low-density lipoprotein (oxLDL) has been shown to be one of the major targets of autoantibodies in atherosclerosis (48, 49). Interestingly, T cells from atherosclerotic plaques proliferated and secreted cytokines in response to oxLDL (50, 51). Bearing that in mind, it is not surprising that large aggregates carrying modifications within the primary structure have the ability to break toleranceIn contrary to that, we believe that this material serve as positive control capable of tolerance reversal in the described human IgG tolerant mouse model.

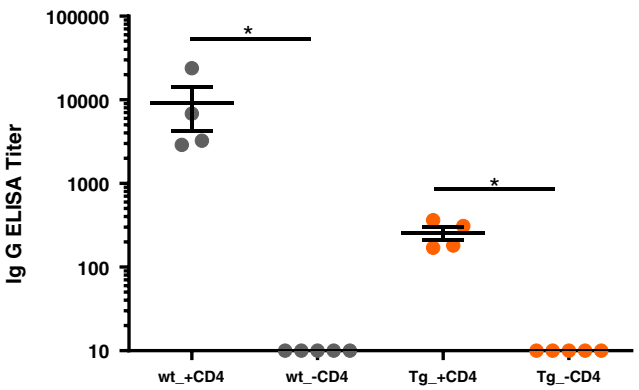

Fig. 7 ADAs to light-stress induced huMAb-1 aggregate require CD4 T cell help. WT and Tg_L17 were treated with anti-CD4 depleting Ab and immunized with light-stress induced huMAb-1 aggregate as indicated in Fig. S4. Specific IgG responses were determined by ELISA 28 post the first immunization. Data shown represent mean IgG ELISA titers ± SEM of 5 mice/group. *$P < 0.05$.

In addition we found that light-stressed huMab1 monomers were not immunogenic in tg mice, despite bearing comparable amounts of neo-epitopes as the more immunogenic light induced dimers and oligomers. For applying light stress, the SunTest XLS+ (Atlas Material Testing Technology LLC, Illinois, USA) was used fitted with a Xenon light source and a filter system providing ID65 light. The exposure reflected very harsh conditions and was set to the duration of 20 h at 765 W/m^2 which corresponds to 3.4 MLxh and 1458 Wh/m^2. This implies that the mAbs were exposed to three times the standard conditions of visible, and seven times of UV-spectrum as defined in the ICH Q1B. Most biotechnological products would not be in compliance with specifications even after single photostability test according ICH Q1B and are thus labelled "to be protected from direct light". The test conditions are therefore considered severe.

The chosen stress conditions were related to the types of physical stress conditions that an Ab molecule may experience during processing or during storage. However, as the exposure to these conditions exceeded realistic conditions by far and/or aggregates induced by these conditions are depleted from therapeutic mAbs preparations during manufacturing, the stress conditions applied are considered to be highly artificial.

In sum, while soluble dimeric and oligomeric aggregates of huMab-1 induced by significant light-exposure could readily

break tolerance and induce a strong immune response in tg mice, monomeric preparations with the same conditions were not or only weakly immunogenic in tg mice. Possibly, in addition to enhanced antigen sampling, larger aggregates may give rise to neoepitopes affecting enzymatic cleavage during antigen processing and thus modifying the repertoire of T cell epitopes presented on the surface of APC. Additionally, also aggregates with or without covalent modifications as significantly stressed by pH or process, were not able to break tolerance in the tg mice. The use of a single mAb as a model mAb in our studies is of course limiting the general nature of our findings as different antibodies react differently upon stress conditions as shown by Joubert *et al.* (21).

As antigen organization determines immunogenicity (52), it is intuitive to think that aggregates, potent inducers of immune response, also bear an organized structure sufficient to strong crosslink BCRs and thus would, at least in part, induce T cell indepent (TI) Ab responses, as it has been suggested in studies of immunogenicity of human IFNβ aggregates (53). However, the Ab response induced by light-stress induced aggregates was completely dependent on T cell help, both in wt and tg mice. This result clearly suggested that light-stress induced huMab-1 oligomers behave as TD Ags and that the reversal of immune tolerance in tg mice depends on the activation of T cells specific for neo-epitopes as generated by the extensive exposure of huMab-1 to light-stress.

Establishment of tolerance against self-antigens can occur both at the T and B cell level. Self-specific B cells can be deleted in the bone marrow (54, 55) or in the periphery (56) in the presence of membrane-bound Ag. They can also become anergic or unresponsive in the presence of high concentration of soluble Ag (57, 58). Yet, low concentration of soluble Ag rather leads to clonal ignorance (59–61). In contrast, tolerance at the T cell level is readily achieved even in the presence of low Ag amounts (61, 62).

In the present tg mouse model, the self Ag is soluble human IgG at rather low concentrations (micrograms per milliliter). Therefore we believe that specific B cells are unaffected whilst T cells specific for native human IgG are deleted or rendered unresponsive and unable to provide help to B cells. The finding that a small fraction of Abs elicited against light induced aggregates also bind native huMab-1 monomers supports this hypothesis.

In this scenario unmodified huMab-1 aggregates such as pH-induced and process-related dimers and oligomers would bind FcγR and mimic IC thus being internalized, processed and presented by DC (and possibly also by B cells bearing specific Ig receptors). Yet, in the absence of T cells specific for unmodified huMAb-1 epitopes no immune response was generated (Fig. 8a).

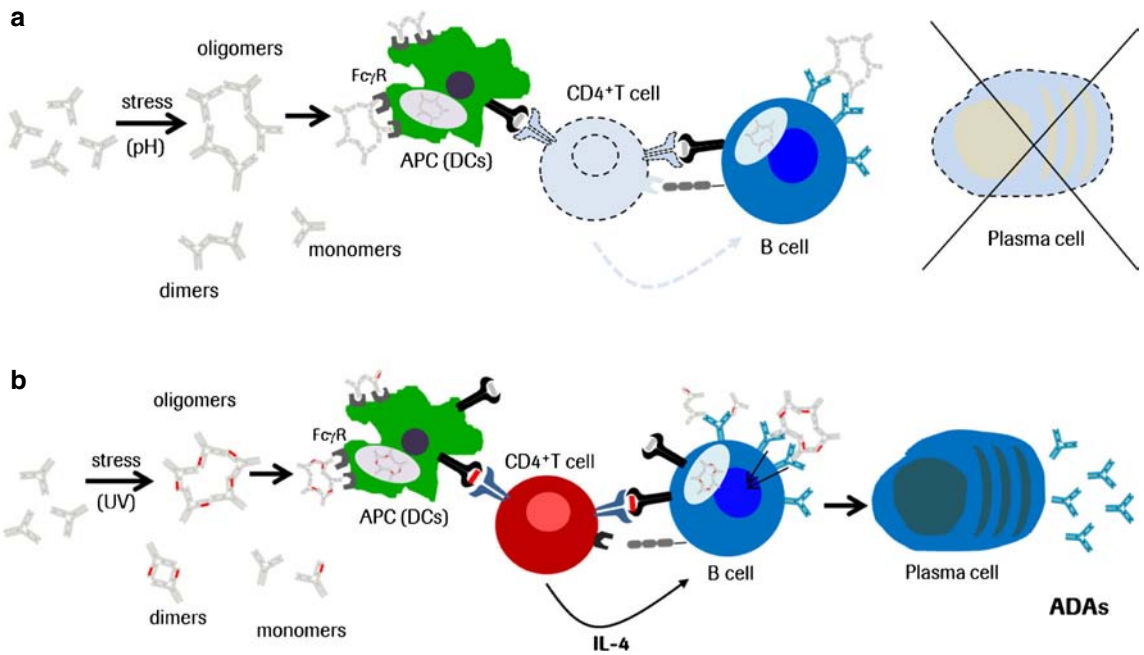

Fig. 8 huMAb-1 aggregates bearing chemical modifications ("neo-epitopes") break immune tolerance and generate ADAs in hIgG1 transgenic mice. Dimers and larger aggregates of hIgG1 mimic immune complexes and bind to FcγR on APCs allowing presentation of both native (*grey*) and chemically modified (*red*) epitopes. B cells specific for both "modified" or native structures of hIgG1 aggregates are not deleted in tg mice. By challenging tg mice with native hIgG1 they will not be activated due the absence of auto reactive T cells specific for native hIgG1 epitopes (*grey*). However, $CD4^+$ T cells specific for "neo-epitopes" (*red*) are not deleted in tg mice and can be activated. Once activated they provide "help" to B cells recognizing hIgG1 leading to plasma cell differentiation and ADAs production.

Upon provision of "neo-epitopes" (chemical amino acid modifications) as generated upon exposure to artificial light-stress, T cell tolerance can be circumvented. Modified dimers and oligomers mimicking IC can cross-link FcγR and are sampled and presented by APCs (and by B cells specific for modified as well as unmodified huMAb-1). The processed peptides containing "neo-epitopes" activate CD4 T cells specific for "neo-epitopes" which in turn become capable of providing help to IgG1-specific B cells which secrete ADAs (Fig. 8b).

CONCLUSION

In summary, we have reported here a novel tg mouse bearing a mini-repertoire of human IgG1 proteins, that is immunologically tolerant to a broad range of human IgG1 molecules. We demonstrated that this mouse model is able to discriminate between immunogenic and non-immunogenic modifications present in aggregated human IgG1. We found that the immunogenic potential of soluble aggregates of human IgG1 strongly depends on the presence of neo-epitopes resulting from harsh stress conditions, i.e. extensive exposure to artificial light. The present model, in combination with other approaches, could aid the assessment of potentially immunogenic modifications in therapeutic Ab preparations.

While the stressed material contained a panel of different degradants, the exact analysis of what has caused immunogenic reactions in this model is not yet possible. Further research will focus on fractionating different species in artificially stressed material as well as to research artificially oxidized material in greater depth in order to better understand the possible relation to immunogenicity in our tg mouse model.

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