

ORIGINAL ARTICLE

The human G1m1 allotype associates with CD4⁺ T-cell responsiveness to a highly conserved IgG1 constant region peptide and confers an asparaginyl endopeptidase cleavage site

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The human G1m1 allotype comprises two amino acids, D12 and L14, in the CH3 domain of IGHG1. Although the G1m1 allotype is prevalent in human populations, ~40% of Caucasoids are homozygous for the nG1m1 allotype corresponding to E12 and M14. Peptides derived from the G1m1 region were tested for their ability to induce CD4⁺ T-cell proliferative responses *in vitro*. A peptide immediately downstream from the G1m1 sequence was recognized by CD4⁺ T cells in a large percentage of donors (peptide CH3_{15–29}). CD4⁺ T-cell proliferative responses to CH3_{15–29} were found at an increased frequency in nG1m1 homozygous donors. Homozygous nG1m1 donors possessing the HLA-DRB1*07 allele displayed the highest magnitudes of proliferation. CD4⁺ T cells from donors homozygous for nG1m1 proliferated to G1m1-carrying Fc-fragment proteins, whereas CD4⁺ T cells from G1m1 homozygous donors did not. The G1m1 sequence creates an enzymatic cleavage site for asparaginyl endopeptidase *in vitro*. Proteolytic activity at D12 may allow the presentation of the CH3_{15–29} peptide, which in turn may result in the establishment of tolerance to this peptide in G1m1-positive donors. Homozygous nG1m1 patients may be more likely to develop CD4⁺ T-cell-mediated immune responses to therapeutic antibodies carrying the G1m1 allotype.

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Introduction

The constant regions of immunoglobulin molecules represent highly conserved multi-domain structures that confer specific functional activities. There are five distinct classes of immunoglobulin in humans: IgM, IgD, IgG, IgE and IgA, with the IgG and IgA classes comprising four and two subclasses, respectively (IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2).¹ Immunoglobulins are expressed early during fetal development, and at high concentrations in the serum.² Significant cellular tolerance to immunoglobulin constant regions is present, as would be expected for any highly expressed, conserved protein.^{3–5}

Allotypes of immunoglobulin exist within the γ 1, γ 2, γ 3 and α isotypes of humans.^{6–9} The G1m1 allotype (also referred to as G1m(a)) in the γ 1 isotype is characterized by two amino acids, aspartate and leucine, D12 and L14, according to the IMGT unique numbering in the CH3 of IGHG1 (positions D356 and L358, respectively, in the Eu gamma1 chain).¹⁰ The absence of the G1m1 allotype (hereto referred to as the nG1m1 allotype; also referred to

as nG1m(a)) is characterized by amino acids E12 and M14 also observed in the CH3 domain of IGHG2, IGHG3 and IGHG4 and therefore, represents an isoallotype for these subclasses (Figure 1a). The nG1m1 allotype is typically found in conjunction with the G1m3 (or G1m(f)) allotype, and only in Caucasoids.⁸ The divergence of the G1m1- and nG1m1-containing haplotype likely occurred at the immigration of early humans to the European continent 30 000–35 000 years ago, as assessed by the accumulation of mutations within the haplotypes.¹¹ Immunoglobulin allotypes may be maintained in the population through a currently uncharacterized heterozygous advantage,¹¹ or through adaptations with respect to viral diseases.^{12,13}

The G1m1 allotype in the CH3 domain can represent an antibody epitope *in vivo*.^{14,15} Anti-allotype antibodies have been detected when individuals who do not possess the allotype in their genome are exposed to antibodies that carry the allotypic amino-acid sequence, such as after platelet transfusion or after multiple child-births.^{16–18} However, the presence of novel amino acids in a normally conserved sequence may also function as a CD4⁺ T helper cell epitope, as has been shown in mice.^{19–23} Productive, cognate interactions between immunoglobulin allotype-specific helper T cells and B cells in rodents have been demonstrated *in vitro*.^{24–27} Therefore, a CD4⁺ T helper cell epitope in the constant region of an exogenously administered antibody could potentially

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G1m1 allotype

HuG1 GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY...
HuG2 GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY...
HuG3 GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY...
HuG4 GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY...

nG1m1 allotype

HuG1 GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY...
HuG2 GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY...
HuG3 GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY...
HuG4 GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY...

Figure 1 The G1m1 allotype of human IGHG1 CH3 occurs in a highly conserved region. Amino-acid alignment of human IGHG1, IGHG2, IGHG3 and IGHG4 CH3 domains between positions corresponding to 1.4 to 30 of IGHG3 CH3. The G1m1 allotype amino acids are underlined.

support the development of antibody responses to other novel regions of the protein, as exemplified by the complementarity determining regions, in humans. In such an example, antibodies specific for the G1m1 sequence may be undetectable but a specific CD4+ helper T-cell response could be present. As a number of approved antibody therapeutics contain the G1m1 allotype in their constant regions, we examined the G1m1-containing region of human IGHG1 CH3 for CD4+ T helper epitopes *in vitro*.

Results

Human CD4+ T-cell responses to the CH3_{15–29} region of the G1m1 and nG1m1 allotypes

A set of 14 peptides was constructed based on the sequence of the IGHG1 CH3 domain encompassing amino-acid positions 1.4–29, according to the IMGT unique numbering system¹⁰ (EU numbering 341–373 (Figure 1b, Table 1)). Peptides #1 and #7 were identical in both sets. Peptides #2–6 differed at two amino acids, CH3 D12>E and L14>M (E356D and M358L). All peptides were tested parametrically using a set of 80 community donor samples (Figure 2). The results showed a significant level of proliferative responses, both in the percent of responding donors and in the overall average magnitude of the response, at peptide #7. We confirmed this result using a set of seven peptides corresponding to peptides A1–A7 synthesized by a separate manufacturer and tested with a subset of 50 community donor samples. Again we saw a high percent response rate (38%) and high average stimulation index (3.61) in response to peptide #7. Finally, in a third confirmation of this result, 15-mer peptides synthesized to represent the entire CH2-CH3 Fc region of γ 1 were tested, and the corresponding G1m1 allotype peptide region induced responses in 20% of all donors with an average SI of 2.59 (data not shown). Peptide #7 is completely conserved across all four IgG subclasses, and encompasses amino acids 15–29 of the CH3 domain.

Response to CH3_{15–29} is absent from donors homozygous for the G1m1 allotype

All 80 community donor samples were assessed for their genomic G1m1 status. Of the 80 donor samples, 28 were homozygous for nG1m1 (Figures 3a and b), 23 were homozygous for G1m1 (Figures 3c and d) and 29 were heterozygous (Figures 3e and f). It was clear that

Table 1 Synthetic peptides corresponding to the G1m1 and nG1m1 regions of the IGHG1 CH3 region.

| Peptides | Sequences | Amino-acid numbers |
|----------|-----------------|--------------------|
| 1 | GQPREPQVYTLPPSR | CH3 1.4–11 |
| A2 | REPQVYTLPPSRDEL | CH3 1–14 |
| A3 | QVYTLPPSRDELTKN | CH3 3–17 |
| A4 | TLPPSRDELTKNQVS | CH3 6–20 |
| A5 | PSRDELTKNQVSLTC | CH3 9–23 |
| A6 | DELTKNQVSLTCLVK | CH3 12–26 |
| 7 | TKNQVSLTCLVKGFY | CH3 15–29 |
| 1 | GQPREPQVYTLPPSR | CH3 1.4–11 |
| N2 | REPQVYTLPPSREEM | CH3 1–14 |
| N3 | QVYTLPPSREEMTKN | CH3 3–17 |
| N4 | TLPPSREEMTKNQVS | CH3 6–20 |
| N5 | PSREEMTKNQVSLTC | CH3 9–23 |
| N6 | EEMTKNQVSLTCLVK | CH3 12–26 |
| 7 | TKNQVSLTCLVKGFY | CH3 15–29 |

Amino acid numbering follows the IMGT designation for CH3 (see ref. 10). Bold lettering indicates amino acids impacted by the G1m1 allotype.

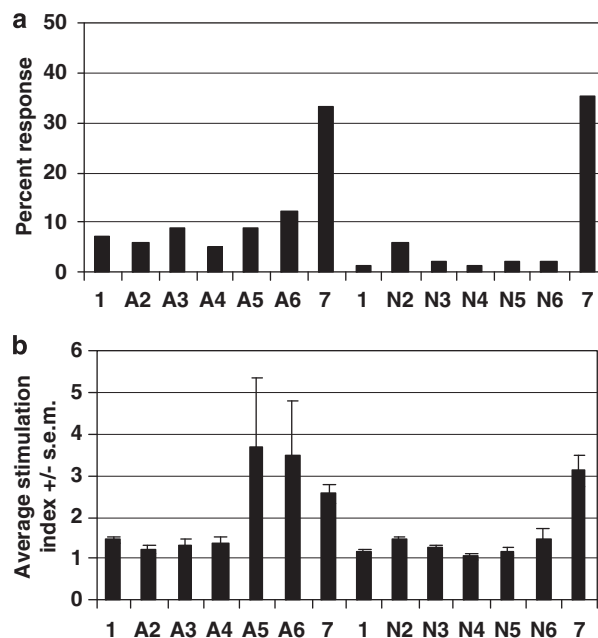


Figure 2 CD4+ T-cell proliferative responses to the G1m1 and nG1m1 peptides. A set of 80 donor samples was tested. A response to a peptide was tabulated if the stimulation index was ≥ 2.95 . (a) Shows the percent responses to the peptides. (b) Shows the average stimulation index \pm s.e.m. for all 80 donors. This result was confirmed in an additional set of 50 donors, and with a second set of peptides from a different manufacturer.

the responses to CH3_{15–29} were occurring in the heterozygous and the nG1m1 homozygous donor samples. In the nG1m1 donors, 12 of 28 were positive for proliferative responses to the peptide, whereas 3 and 4 donors in each test of the peptide were positive for responses in the G1m1 homozygous donors. Heterozygous donors responded at approximately the same rate as the nG1m1 donors. This result suggests that CD4+ T cells from donors with two copies of the G1m1 allotype selectively lack the ability to proliferate in response to CH3_{15–29}.

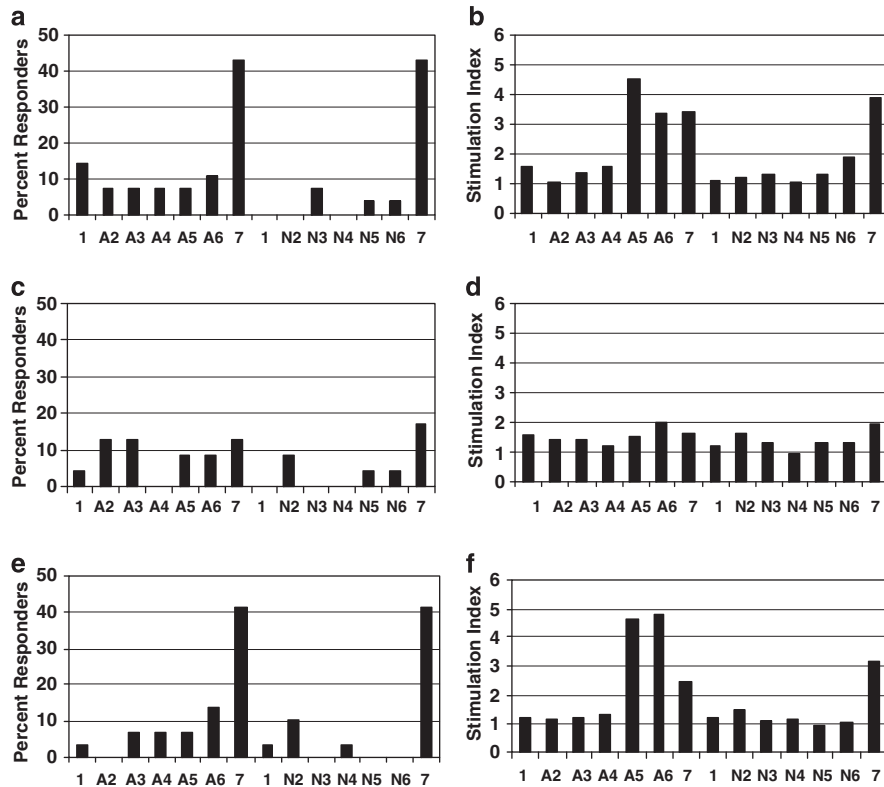


Figure 3 CD4+ T-cell responses to the G1m1 and nG1m1 peptides separated by donor G1m1 status. The data from Figure 2 were segregated according to the G1m1 allotype expression of the donor. (a, b) nG1m1 homozygous donors ($n = 28$). (c, d) G1m1 homozygous donors ($n = 23$). (e, f) Heterozygous donors ($n = 29$).

Heterozygous donors display an intermediate response to CH3₁₅₋₂₉

Both the homozygous nG1m1 and the heterozygous donors responded to CH3₁₅₋₂₉ with a stimulation index of greater than 2.95 with an incidence of ~40%. However, the average magnitude of the response was lower in heterozygous donors (Figure 4a). The difference between the average response rate found with the nG1m1 donors and the G1m1 homozygous donors was highly significant (Student's *t*-test, $P < 0.01$). The difference between the responses of the heterozygous donors and the G1m1 homozygous donors was lower, but did not reach statistical significance ($P = 0.11$). The overall magnitude of the responses mounted by the donor CD4+ T cells suggests a haplotype dose effect in which the lowest responses were shown by the G1m1 homozygous donors, the highest overall responses by the nG1m1 homozygous donors and an intermediate response by the heterozygous donors (Figure 4b). This result suggests that the G1m1 phenotype may be inducing tolerance to the CH3₁₅₋₂₉ sequence, but that tolerance induction is occurring stochastically.

CD4+ T-cell proliferative responses to CH3 region peptides are accompanied by cytokine production

Culture supernatant samples from two donors were tested for the presence of cytokines on day 5 (Figure 5). The donor in Figure 5a was homozygous for the nG1m1 allotype and is a robust responder to CH3₁₅₋₂₉ and to the G1m1 sequence-containing peptides immediately upstream (peptides A5 and A6). Interleukin (IL)-13 and

IL-2 are detectable in all the three peptide responses. Interferon (IFN)- γ is present in the responses to CH3₁₅₋₂₉ and peptide A5, but not A6. Consistent with the low level of proliferation, negligible cytokine responses are seen in the N-peptide series. The donor in Figure 5b was heterozygous for the G1m1 allotype and responded poorly to all of the peptides, mounting only a modest proliferative response to CH3₁₅₋₂₉. Consistent with this reduced response, small amounts of IL-2 are found in the CH3₁₅₋₂₉-containing wells.

All supernatants were found negative for IL-4, IL-5, IL-10, IL-12p70, tumor necrosis factor (TNF)- α , IL-6, IL-1, RANTES and MCP-1. The presence of IL-2, IL-13 and IFN- γ suggests that the CD4+ T cells responding to CH3₃₅₉₋₃₇₃ display an intermediate, or Th0, phenotype. They are not classifiable as either Th1-like or Th2-like even though they are expressing both Th1-associated (IFN- γ) and Th2-associated (IL-13) cytokines.

HLA-DR associations with responses to the CH3 peptides

All 80 donors were also HLA-DRB1 and HLA-DQB1 typed. No significant correlations were found between the HLA class II genotype of the donor and response rate to any peptide in the set. However, the magnitude of the proliferative response to CH3₁₅₋₂₉ was higher in nG1m1 donors who were also HLA-DRB1*07+ (average SI = 4.87 ± 1.11 vs 2.11 ± 0.23 ; $P < 0.03$; Figure 6). Interestingly, the effect of HLA-DRB1*07 and nG1m1 genotypes on the response to CH3₁₅₋₂₉ were synergistic, in which the expected response rate of the HLA-DRB1*07+ responders and the nG1m1 responders were not simply

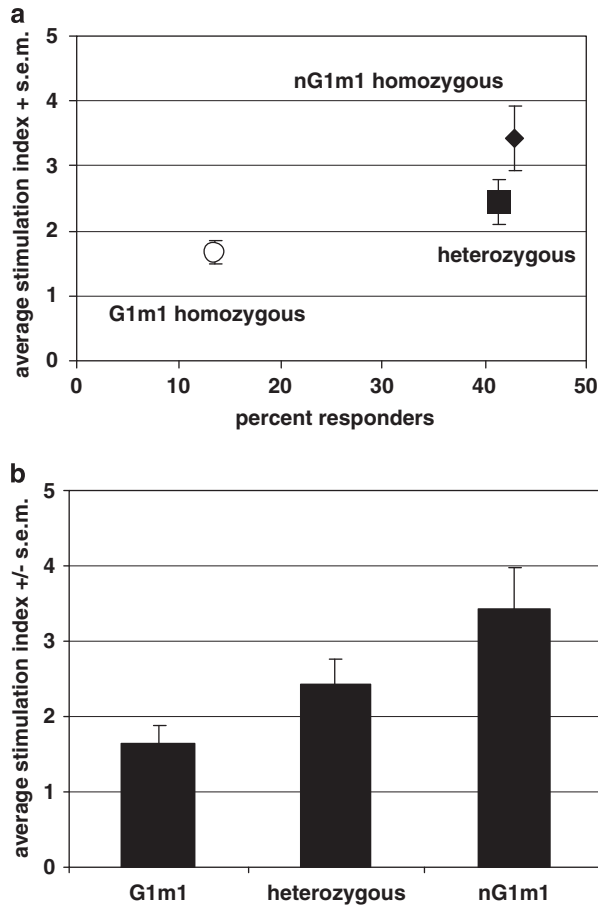


Figure 4 G1m1/nG1m1 heterozygous donors display an intermediate response pattern to the CH₃_{15–29} peptide. (a) The percent responders and the average stimulation index for the G1m1 homozygous (open circle), nG1m1 homozygous (black diamond) and heterozygous donors (black square) are shown plus or minus the s.e.m. of the stimulation index response. (b) The response rates of the G1m1 homozygous, nG1m1 homozygous and heterozygous donors are shown.

additive. The effect is not additive because taking G1m1 homozygous/HLA-DRB1*07 negative donor responses as a reference (mean SI=2.11), G1m1 homozygous/HLA-DRB1*07 positive donor responses have an SI that is 2.8% lower (mean = 2.05). The nG1m1 homozygous/HLA-DRB1*07 negative donor responses display an average SI that is 18.5% higher than responses from G1m1 homozygous/HLA-DRB1*07 negative donors (mean SI = 2.50). If the effects were additive, we would expect that nG1m1 homozygous/HLA-DRB1*07 responses to have a mean stimulation index 15.7% (18.5 ± 2.8%) higher than the reference group. Instead, these donors have a mean stimulation index that is 130% higher than the reference group (mean SI = 4.87; Figure 6). This non-additive influence is statistically significant ($P < 0.02$). This analysis suggests that the nG1m1, HLA-DRB1*07 homozygous donors carry CD4⁺ T cells capable of recognizing and proliferating in response to a peptide present in the CH₃ region of the γ 1 isotype, and that heterozygous and G1m1 homozygous HLA-DRB1*07 donors contain CD4⁺ T cells that are specifically not responding to this peptide.

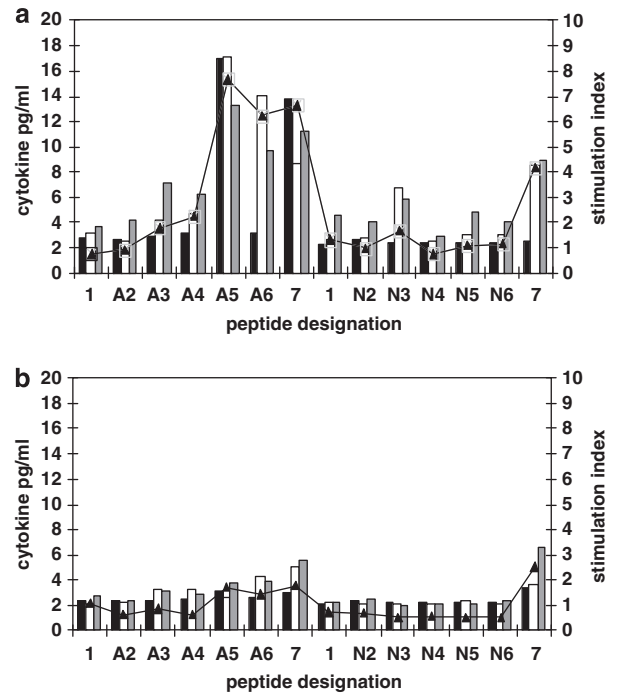


Figure 5 CD4⁺ T-cell cytokine responses to the CH₃ allotype-region peptides. Supernatants from proliferative responses of CD4⁺ T cells co-cultured with autologous dendritic cells and allotype region peptides were tested for the presence of human cytokines by a multiplex assay. Black bar: IFN- γ ; white bar: IL-13; gray bar: IL-2. CD4⁺ T-cell proliferative response to each peptide is shown by the black triangles. Donor in (a) was homozygous for nG1m1. The donor in (b) was heterozygous for G1m1.

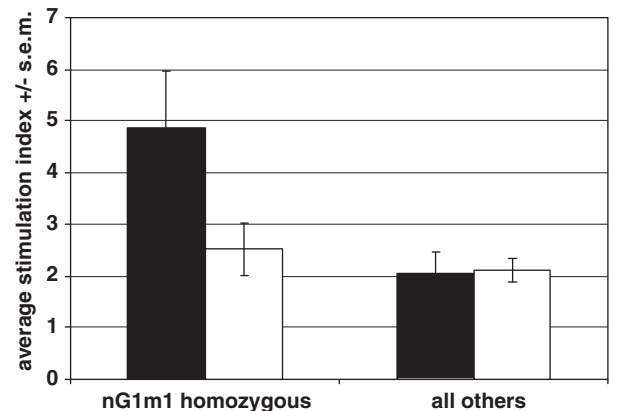


Figure 6 HLA-DRB1*07⁺ and nG1m1 homozygous donor CD4 T-cell responses to CH₃_{15–29}. Average stimulation index values for donors positive for HLA-DRB1*07 at one allele (black bars) were compared with all other donor results (open bars). Donors who were nG1m1 homozygous and HLA-DRB1*07 positive displayed significantly higher average stimulation indexes than all other combinations ($P < 0.03$). HLA-DRB1*07⁺/nG1m1 homozygous $n = 11$. HLA-DRB1*07⁻/nG1m1 homozygous $n = 17$. HLA-DRB1*07⁺/other allotype $n = 14$. HLA-DRB1*07⁻/all other allotype $n = 38$.

CD4⁺ T-cell responses to G1m1 allotype-containing Fc fragments from commercial antibodies

Fc fragments were purified from commercially available bevacizumab, infliximab and adalimumab therapeutic

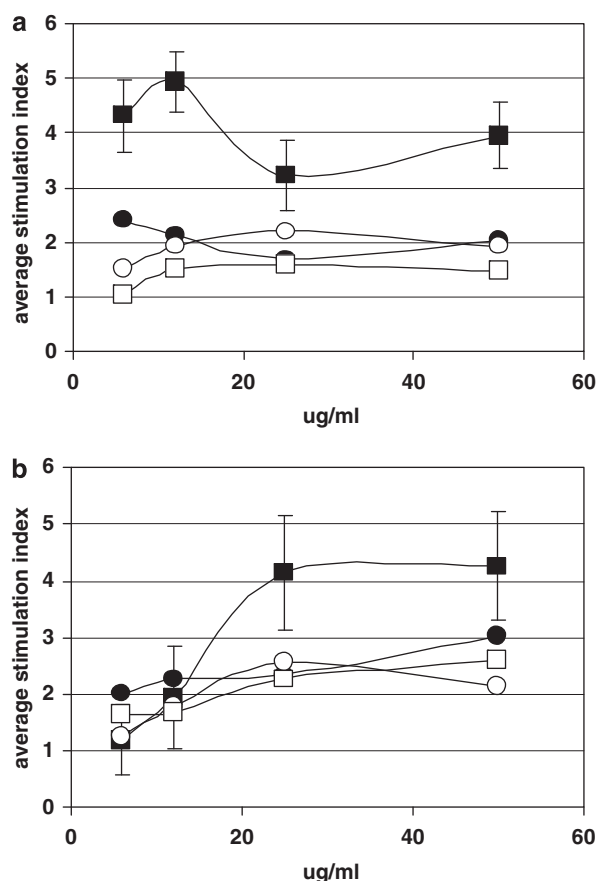


Figure 7 CD4+ T-cell proliferative responses to G1m1 allotype-containing Fc fragments. The G1m1-containing Fc fragment purified from infliximab after papain cleavage (closed symbols) and the nG1m1-containing Fc fragment purified from bevacizumab after papain cleavage (open symbols) were co-cultured with CD4+ T cell-enriched PBMC from G1m1 homozygous (circles) or nG1m1 homozygous (squares) donors. (a) Fc fragments were titrated into cultures as soluble proteins. (b) Fc proteins were heat-denatured for 5 min at 95 °C then cooled to room temperature. Proliferation was assessed by tritiated thymidine incorporation on day 6. s.e.m. is only shown on the infliximab Fc fragment proliferative responses for clarity.

antibodies. Infliximab and adalimumab contain a $\gamma 1$ constant region that carries the G1m1 allotype, whereas bevacizumab contains a $\gamma 1$ constant region that was modified to carry the nG1m1 allotype. These two antibodies are expressed in mammalian cells but from different species; infliximab is produced in a murine SP2/0 cell line, whereas bevacizumab is expressed in CHO cells. The use of different expression cell lines could lead to differences in the glycosylation patterns of the two Fc fragments. Peripheral blood mononuclear cell (PBMC) from a series of donors homozygous for nG1m1 or G1m1 were tested *in vitro* for proliferative responses to the two different allotype-carrying Fc proteins (Figure 7). A total of five G1m1 homozygous donors and four nG1m1 homozygous in-house donors were tested. The Fc proteins were tested as soluble proteins (Figure 7a), or in a heat-denatured format (Figure 7b). The results were similar for both protein formats. PBMC from donors homozygous for nG1m1 proliferated more strongly in the presence of the G1m1 + Fc protein than the nG1m1

Fc protein. PBMC from donors who were G1m1 homozygotes did not respond to either of the Fc proteins. This result suggests that the G1m1 allotype sequence is potentially immunogenic in nG1m1 donors and implies tolerance induction in the donors who carry the G1m1 haplotype. We did not consider the presence or absence of the G1m2 (or G1m(x)) allotype in these experiments.

Processing of the Fc fragment of human IGHG1 by asparaginyl endopeptidase reveals a proteolytic clip site at the G1m1 sequence

We reasoned that the unique amino-acid sequence at the G1m1 allotype might represent a proteolytic cleavage site that could alter the processing and presentation of the $\gamma 1$ constant region. Presentation of the CH3_{15–29} fragment could result in tolerance induction or deletion of CD4+ T cells with this specificity. Alternatively, a unique clip site could result in modified processing such that the CH3_{15–29} peptide fragment is not presented. Asparaginyl endopeptidase (AEP; legumain) is a proteolytic enzyme active in the antigen-processing pathway.²⁸ Human AEP has been demonstrated to cleave immediately after both asparagine and aspartic-acid residues during autocatalysis to its fully functional mature form.^{29,30} As the G1m1 amino-acid modification contains an aspartic-acid residue at position D12, we performed an *in vitro* proteolysis experiment using purified recombinant human AEP. The AEP cleavage assay was performed on both purified Fc fragments (Figure 8a lanes 2 and 4) and intact IgG molecules (Figure 8a lanes 1 and 3). We performed the assay on Fc and IgG proteins that were constructed to be identical except for the presence or absence of the G1m1 allotype in the CH3 of the constant region. After digestion with AEP, we noted a fragment at ~3.5 kDa in the G1m1-carrying Fc and IgG protein digests (fragment d, lanes 1 (IgG) and 2 (Fc)) that was not apparent in the nG1m1 Fc or IgG reactions (lanes 3 (IgG) and 4 (Fc)). The protein fragments labeled b (~4 kDa) were apparently identical in both the G1m1 and nG1m1 Fc digests, and N-terminal sequencing showed that the fragment b amino-acid sequence initiated at CH2 A78. Fragments c and d were found to have identical N-terminal sequences that initiated at CH2 K109, but were different in size by ~500 Da. Our interpretation of this data is that the carboxy terminal ends of the protein fragments c and d were different and that the size difference was consistent with an AEP-mediated cleavage event at the approximate site of the G1m1 amino-acid modification. Fragment a is the result of an initial clip at N326 that cleaves the Fc protein approximately in half (12.4 and 13.6 kD fragments). The putative AEP cleavage sites in the $\gamma 1$ constant region were reconstructed from the N-terminal sequencing data and from the molecular weight of the Fc-derived fragments (Figure 8b).

Discussion

We tested a set of peptides encompassing the G1m1 allotype of human IGHG1 CH3 in human CD4+ T-cell proliferation and cytokine secretion assays. We found that the CH3_{15–29} region induced proliferative and cytokine responses in ~30% of all community donors tested. We confirmed this result in two additional tests with newly synthesized peptides and in discrete random

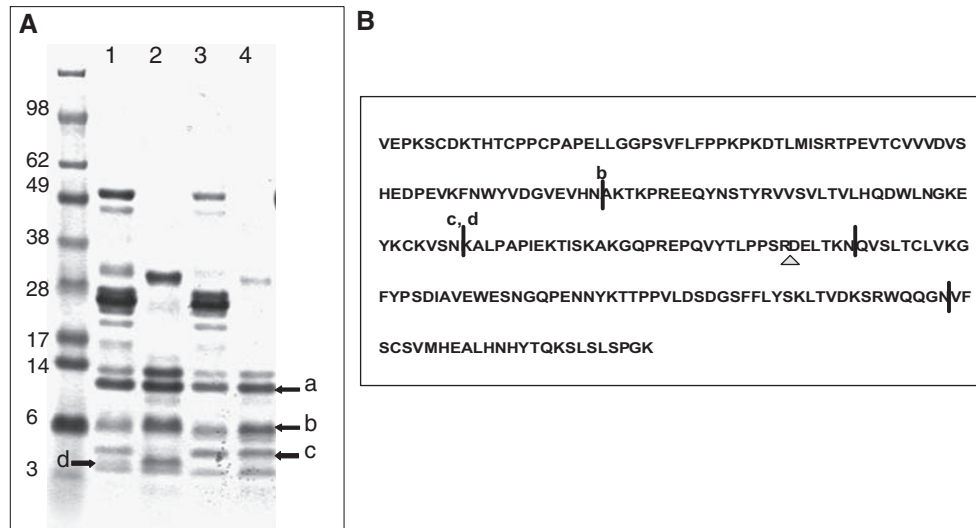


Figure 8 The G1m1 allotype creates a novel AEP cleavage site. (A) Sodium dodecyl sulfate-PAGE of AEP cleavage of G1m1- and nG1m1-containing Fc and IgG1 proteins. Lane 1: AEP-treated IgG1-G1m1 antibody. Lane 2: AEP-treated Fc-G1m1 fragment. Lane 3: AEP-treated IgG1-nG1m1 antibody. Lane 4: AEP-treated Fc-nG1m1 fragment. Molecular weight markers are shown in kD. (a-d) fragments excised for N-terminal sequencing. (B) Proposed AEP cleavage sites in the CH2-CH3 domains of the IGHG1 isotype. The labeled black bars indicate N-terminal regions of sequenced protein fragments. The triangle indicates the approximate position of the proposed G1m1-specific cleavage site.

donor sets. Responding donors carried at least one allele of the nG1m1 allotype in their genome. The CH3₁₅₋₂₉ peptide sequence may therefore represent a CD4⁺ T-cell epitope in a specific subset of human donors. This result suggests that IgG1-based therapeutic antibodies that carry the G1m1 allotype in their CH3 regions may in fact contain a CD4⁺ T helper epitope. The presence of a CD4⁺ T-cell epitope in the Fc region of a therapeutic antibody may both provoke and support a T-dependent anti-idiotype response to the therapeutic.³¹

We were surprised to find a CD4⁺ T-cell epitope not in the allotype-containing CH3₁₂₋₁₄ region, but rather immediately downstream of it. On further investigation, CD4⁺ T-cell proliferative and cytokine responses to the conserved peptide region were found only in cells from donors carrying the nG1m1 allotype. Donors homozygous for G1m1 did not respond to the CH3₁₅₋₂₉ peptide above background rates. In addition, we found that the allotype amino acids confer a unique AEP cleavage site in G1m1⁺ immunoglobulin molecules. These results show that the tolerance is induced to this conserved peptide only in G1m1 homozygous subjects, and nG1m1⁺ donors are incompletely tolerized *in vivo* and therefore, mount proliferative responses to the epitope peptide. As a similar percent of heterozygous donors as the nG1m1 homozygous donors are mounting responses to the CH3₁₅₋₂₉ peptide (~40% for both sets of donors), but their overall magnitude of response is lower, our hypothesis is that only the high affinity CD4⁺ T cells specific for CH3₁₅₋₂₉ are deleted in heterozygous donors due to a lower 'dose' of the processed peptide. In G1m1 homozygous donors, most CD4⁺ T cells with specificity for the CH3₁₅₋₂₉ peptide are functionally deleted because of the increased relative dose of the peptide *in vivo*. An alternative hypothesis is that responses to the CH3₁₅₋₂₉ peptide are secondary responses in nG1m1 donors, and G1m1 homozygous donors do not carry secondary T-cell responses to the epitope as AEP processing destroys

epitope presentation.³² If processing destroys the epitope, the nG1m1 Fc protein should provide an intact epitope for recognition by CD4⁺ T cells from G1m1 homozygous donors. Our *in vitro* proliferation data with Fc-derived proteins do not support this hypothesis. The corollary to this conclusion is that G1m1 donors' non-responsiveness to the Fc-derived proteins is therefore due to tolerance induction, which supports our hypothesis. That human constant regions are subjected to processing and presentation has been described,³³ and presentation of human G1m1 allotype-associated peptides is consistent with data from mouse models where murine allotypes can be encountered as antigenic proteins.^{3,22} Finally, this hypothesis is consistent with the demonstration that changes of amino acids in the sequence of constant regions can result in an immunogenic Fc-based protein construct.³⁴⁻³⁶

Homozygosity of the nG1m1 allotype occurs only in Caucasoids, and at a rate of ~40% (see ref. 8) The remaining 60% are heterozygous, or homozygous for the G1m1 allotype. The G1m1 allotype is found at 100% of the population in all other ethnicities. It would be logical, therefore, to construct humanized therapeutic antibodies using the most prevalent allotype. However, our result suggests that administering an IgG1 therapeutic antibody carrying the G1m1 allotype might provoke a CD4⁺ T-cell response in nG1m1 subjects, similar to responses to allotypes observed in mice and rats.^{19,25-27} A T helper response directed at the constant region could be akin to the 'carrier protein' situation, where B cells with specificity for the idiotype of the therapeutic could receive immunological help for the Fc-specific CD4⁺ T cell.

At the present time there is little published data from human clinical studies characterizing the allotype status of patients who develop human anti-human(ized) antibody (HAHA) responses.^{37,38} Both of these published studies tested for antibody development to chimeric

antibodies. The first chimeric and humanized antibodies were constructed using an IGHG1 constant region gene sequenced, and identified in 1982 that contained the G1m1 allotype.^{39,40} These included alemtuzumab, an anti-CD52 monoclonal antibody, infliximab, an anti-TNF- α antibody, huAb A33, a complementarity determining regions-grafted anti-human A33 monoclonal antibody and the fully human monoclonal antibody derived by phage display, adalimumab.⁴¹ All of these antibodies induced HAHA in a significant number of patients. Allotype analysis of HAHA-producing patients in an early alemtuzumab rheumatoid arthritis trial suggested that allotype differences did not correlate with the development of HAHA as even allotype-matched patients were capable of expressing antibodies against alemtuzumab.³⁸ However, the patient sample size was quite small and this observation does not preclude the development of anti-allotype helper cells in some patients. The recent study of infliximab-treated patients found no statistical correlation between G1m1 allotype and the development of antibodies to infliximab (ATI). However, reanalysis of the frequency of heterozygous and nG1m1 homozygous donors as a group showed a comparative enrichment for the nG1m1 allotype in the ATI+ and the high titer ATI+ donors as compared to ATI negative, and the control sample distributions.³⁷ No anti-constant region-specific antibodies were detected in huAb A33-treated patients.⁴² This result does not address whether allotype-specific helper cells supported the development of the HAHA response. Finally, the fully human antibody adalimumab can induce neutralizing antibody responses in a significant number of patients.⁴³ HAHA responses to adalimumab have not been fully characterized as yet, but the majority of the response is likely to be directed at the V domain, as neutralization of efficacy is a consequence of HAHA development.⁴⁴ It would be of distinct interest to characterize the allotype status of human patients generating anti-human antibodies to adalimumab as the overt immunogenicity of the infliximab, and alemtuzumab variable domains may have overshadowed any contribution from putative allotype-specific helper T cells.

Interestingly, during the humanization of the anti-P185^{HER2} murine monoclonal antibody, the human IGHG1 isotype was modified from G1m1 to nG1m1, creating the non-natural haplotype G1m17, nG1m1 (or G1m(z, -)) present in trazutuzumab.⁴⁵ Although it is difficult to compare the relative immunogenicity of various therapeutic antibodies, the trastuzumab antibody molecule has not induced measurable immune responses in many breast cancer patients.⁴⁶ It would be of significant interest to understand whether allotype-specific helper T cells in certain individuals were responsible for the development of neutralizing HAHA reactions, as these drugs can be efficacious but are very expensive to administer.⁴⁷ In this respect, the G1m1 status of patients could be a potential biomarker for HAHA development.

In conclusion, we show that the G1m1 allotype of the IGHG1 isotype of human antibodies associates with a CD4+ proliferative and cytokine response in human donors homozygous for the nG1m1 allotype. Proteolytic cleavage at the allotype amino acids may reveal a novel epitope in G1m1 donors, which in turn may result in epitope-specific tolerance induction only in G1m1

positive donors. If our observation *in vitro* holds true for exogenously administered antibodies in humans, the G1m1 status of patients could be screened before administration of G1m1-carrying antibodies. Finally, our findings may be of relevance to the engineering of future antibody therapeutics to reduce the human immune responses elicited to these proteins.

Materials and methods

Peptides

All peptides were manufactured as consecutive 15-mers overlapping by 12 amino acids (Table 1). Two separate sets were tested and the data were compiled. One set was synthesized by Pepsan Systems (Lelystad, The Netherlands), and a second set by Mimotopes (Melbourne, Victoria, Australia). Peptides were pin-synthesized, cleaved and lyophilized. On arrival the peptides were resuspended in DMSO at $\sim 2\text{ mg ml}^{-1}$. After resuspension, the peptide stocks were kept at -80°C .

Proteins

The Fc fragment (hinge-CH2-CH3) of various human IgG1 commercial and in-house antibodies was purified after treatment with immobilized papain (Pierce Chemicals, Rockford, IL, USA). Briefly, antibodies were incubated at 37°C with immobilized papain as per manufacturer's instructions for 4 h to overnight. Proteolysis was assessed by high-pressure liquid chromatography. Fc fragments were purified from the protease-treated antibodies by immobilized protein A-sepharose (Pierce Chemicals). Purity was assessed by sodium dodecyl sulfate-PAGE. Purified Fc was dialyzed against phosphate-buffered saline overnight, and was filter sterilized. Endotoxin levels were monitored by LAL assay (Endosafe, Charles River, Wilmington, MA, USA) and were less than 1 EU per mg protein.

CD4+ T-cell proliferation assays

PBMC samples were drawn from community donors at the Stanford Blood Center (Palo Alto, CA, USA). The CD4+ T-cell proliferation assay was performed as described.^{48,49} Briefly, dendritic cells were differentiated from human peripheral blood monocytes by culture in AIM V media (Invitrogen, Carlsbad, CA, USA) plus GM-CSF and IL-4 for 5 days. Dendritic cells were activated by the addition of IL-1 and TNF- α and then collected for use on day 7. Autologous CD4+ T cells were isolated by negative selection from thawed PBMC samples (Stem Cell Technologies, Vancouver, British Columbia, Canada) then co-cultured with irradiated dendritic cells and peptides ($5\ \mu\text{g ml}^{-1}$) in AIM V media for 5 days. CD4+ T cells were used at 2×10^5 cells per well, with 2×10^4 dendritic cells as antigen-presenting cells. Proliferation was assessed on day 6. A donor was considered a responder for a specific peptide if the average proliferation of the experimental wells was 2.95 times higher than the control DMSO only wells. This cut-off value has been validated to return the highest sensitivity of positive versus false positive responses.²¹ Validation of the cut-off value was assessed by performing CD4+ T-cell proliferation assays using peptides derived from a number of well-characterized protein antigens, such as staphylokinase and HPV E6 and E7 and

comparing the results with published T-cell epitope mapping results. The positive control for the assay was tetanus toxoid included in 2–4 replicate wells at 1.25 µg ml⁻¹. The tetanus toxoid positive control must reach a stimulation index of at least 3.0 for the peptide proliferation results to be included in the database. All assay wells contained 0.25% DMSO, including the tetanus toxoid positive controls and the no-peptide negative controls.

Cytokine assays

On day 5 of incubation, 20 µl samples were removed from the cell culture wells and replicates were pooled. Human cytokine levels were determined using the Upstate Beadlyte 12-plex system (Millipore, Billerica, MA, USA). Analytes tested were IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IFN-gamma, TNF-alpha, RANTES and MCP-1. Data were analyzed on a Luminescence-100 device (Luminex, Austin, TX, USA).

Low-resolution HLA class II typing and G1m1 allotype determination

HLA class II typing was performed using the Dynal SSO PCR-based system (Invitrogen). The G1m1 allotype of donors was determined by nested PCR amplification followed by genomic sequence analysis. First, a 503 bp fragment containing the CH3 region of IgG was amplified from donor genomic DNA using the Expand High Fidelity PCR System (Roche, Indianapolis, IN, USA) with IgG class-specific forward primer 5'-ATGGA CAGAGGCCGGCTCG-3' and reverse primer 5'-GTGC TTTATTTCATGCTGGG-3'. A total of 35 cycles of amplification (30' at 95 °C, 30' at 65 °C, 45' at 72 °C) were performed in the buffer provided by the manufacturer. Next, a 414 bp fragment containing the G1m1 allotypic sequence of IgG1 was amplified from the first PCR product using the Expand High Fidelity PCR System with IgG1 subclass-specific forward primer 5'-AGAGT GACCGCTGTA-3' and reverse primer 5'-AAGCATCC TCGTGCGACCG-3'. Furthermore, 35 cycles of amplification (30' at 95 °C, 90' at 72 °C) were performed in Buffer D (Invitrogen). Finally, PCR products were sequenced on an ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA). Homozygosity and heterozygosity were apparent by an analysis of the sequencing traces.

PBMC Fc protein proliferation assays

Peripheral blood samples were obtained from G1m1-typed in-house donors. PBMCs were isolated by density centrifugation, and monocytes and CD4+ T cells were enriched by positive selection with anti-CD4+ magnetic particles (Stem Cell Technologies). CD4+ enriched PBMCs were cultured at 2 × 10⁵ cells per well in AIM V media. Sterile, endotoxin-free Fc fragment proteins were titrated into the wells, and the cultures were incubated for 5 days. Proliferation and IFN-γ secretion were assessed on day 6. Fc fragment proteins were tested either as soluble protein, or after heat denaturation at 100 °C for 5 min.

Asparaginyl endopeptidase activity assay

Purified IgG1 antibodies from constructs containing either the G1m1 or nG1m1 mutation were treated with immobilized papain (Thermo Scientific, Rockford,

IL, USA) and the Fc fragments were purified using immobilized protein A (Thermo Scientific). Fc digestion was performed in 50 mM sodium acetate, 100 mM sodium chloride, pH 4.0, with 5 mM DTT and 175 units of recombinant human legumain (AEP, R and D Systems, Minneapolis, MN, USA) per 1 µg of protein. The reaction was allowed to progress overnight at 37 °C. The digested protein was separated on sodium dodecyl sulfate-PAGE gels, and the appropriate bands were excised for N-terminal sequencing (Commonwealth Biotechnologies, Richmond, VA, USA).

Conflict of interest

All of the authors of this manuscript are employed in the biotechnology industry, at companies that are developing therapeutic monoclonal antibodies.

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