FEBS Letters 495 (2001) 82–86 FEBS 24772

The G protein β3 subunit 825T allele is a genetic marker for enhanced T cell response

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Received 26 January 2001; revised 13 March 2001; accepted 15 March 2001

First published online 21 March 2001

Edited by Masayuki Miyasaka

Abstract The G protein β3 subunit (*GNB3*) 825T allele is predictive of enhanced Gi protein activation. Studying the influence of C825T allele status on cellular in vitro immune responses towards recall antigens and interleukin-2 stimulation we observed a 2–4-fold, significantly increased proliferation in homozygous 825T (TT) vs. C825 allele (CC) carriers. Furthermore, lymphocyte chemotaxis and $CD4^+$ T cell counts of individuals with TT+TC genotypes were significantly enhanced compared to the CC genotype. In summary, it appears that C825T allele status is highly predictive of immunocompetence and could be a candidate gene in disorders associated with inadequate immune response.

Key words: Lymphocyte; Proliferation; Chemotaxis; CD4; G protein; Genotype

1. Introduction

Heterotrimeric G proteins are important mediators of intracellular signal transduction not only for heptahelical G protein-coupled receptors but also for some receptors with intrinsic tyrosine kinase activity. Initiating signals for G proteins include the binding of hormones, chemokines, and neurotransmitters. Recently we described a C825T polymorphism in the gene *GNB3* encoding the β3 subunit of heterotrimeric G proteins [1]. The 825T allele is associated with the generation of a functionally active splice variant, Gβ3-s, which is expressed in cells from 825T allele carriers [1]. The 825T allele is predictive of enhanced activation of pertussis toxin (PTX)-sensitive G proteins as seen from enhanced chemotaxis of human neutrophils towards *N*-formyl-methionyl-leucyl-phenylalanine or interleukin (IL)-8 [2,3]. Transient expression of Gβ3-s in COS-7 cells enhances chemotaxis which underscores the functional importance of this splice variant in enhancing G protein activation [3]. As there were hints for an influence of *GNB3* genotype on innate cellular immunity (chemotaxis of neutrophils), we speculated that regulation of the specific cellular immune response may also be affected by *GNB3* genotypes.

To investigate if *GNB3* genotype modulates specific cellular immune response, lymphocyte transformation after stimulation with common recall antigens, and IL-2 as well as lymphocyte chemotaxis and also lymphocyte subpopulations were analyzed.

2. Materials and methods

2.1. Study subjects

Venous blood samples were obtained from healthy, male, non-smoking individuals selected for TT, TC, and CC genotypes ($n = 63$ for lymphocyte transformation tests (LTT), $n = 35$ for IL-2-induced proliferation, $n = 23$ for PTX inhibition assays, $n = 10$–30 for chemotaxis assays (depending on the experimental setting), and $n = 202$ for enumeration of leukocytes by flow cytometry). Besides, non-selected healthy medical staff and students (27 female, 25 male) were booster-vaccinated against hepatitis B virus (HBV, Gen H-B-Vax, Chiron Behring, Marburg, Germany) on average 1.5 years after their last immunization and heparinized blood was taken 1 week after booster shot to perform an HBV-specific LTT.

2.2. DNA genotyping

Genomic DNA was extracted from EDTA-blood with a proprietary reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. *GNB3* C825T allele status was determined after PCR amplification and restriction analysis as described [1].

2.3. Lymphocyte proliferation in response to recall antigens, mitogens, and IL-2

Cellular in vitro responses towards 12 recall antigens (six different concentrations each) and the mitogens phytohemagglutinin (PHA, 0.5–4 μg/ml), concanavalin A, (5–40 μg/ml), pokeweed mitogen (0.6–5 μg/ml), and anti-CD3 (OKT3, 0.03–0.25 μg/ml) were quantified using standardized assay formats (LT2 antigen, No. 7051, BAG, Lich, Germany, LTT mitogen: in-house prepared) as described previously [4]. In a separate assay, cells were stimulated with IL-2 (25 and 100 U/ml, Proleukin, Chiron, Ratingen, Germany). In brief, peripheral blood mononuclear cells (PBMCs) from heparinized blood were collected after Ficoll-Paque™ density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) centrifugation and 50 000 cells were incubated with antigens, mitogens, and IL-2, respectively, in 200 μl of cell culture medium (RPMI 1640, Gibco, Life Technologies, Paisley, UK, with 10% of inactivated pooled human serum, Institute of Transfusion Medicine, University Hospital, Essen, Germany) per well of microtiter plates (37°C, 5% CO_2). Mitogen cultures were set up as quadruplicates for 3 days. Antigen and IL-2 cultures were grown in sixtuplicates

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Abbreviations: cpm, counts per minute; Gi protein, inhibitory G protein; *GNB3*, G protein β3 subunit; HBV, hepatitis B virus; HPF, high power field; HSV, herpes simplex virus; IL-2, interleukin-2; LTT, lymphocyte transformation test; MFI, mean fluorescence intensity; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; PPD, tuberculin; PTX, pertussis toxin; SDF-1α, stromal cell-derived factor 1α; S.E.M., standard error of the mean

PII: S0014-5793(01)02339-0

for 5 days and triplicates for 6 days, respectively. For the last 16 h, the cultures were labeled with 1 μCi [^{3}H]thymidine per well (TRA.120, specific activity 5 Ci/mmol, Amersham, Buckinghamshire, UK). The cultures were then harvested (Micro96 Harvester, Skatron Instruments, Transby, Norway) onto glass fiber filtermates (Wallac, Turku, Finland) and the incorporated radioactivity was quantified by liquid scintillation counting (1205 Betaplate, Wallac). For evaluation, the second highest [^{3}H]thymidine uptake of antigen and IL-2 cultures was chosen. In addition, each antigen concentration was considered separately. Increment counts per minute (cpm) values were generated as antigen and IL-2 proliferation minus autologous (unstimulated) proliferation. In some experiments, PBMCs (5×10^6/ml) were pre-incubated with or without PTX (100 ng/ml) for 24 h in RPMI 1640 plus 10% pooled inactivated human serum. Thereafter, determination of lymphocyte proliferation in response to recall antigens, and IL-2 was performed as described above.

Furthermore, cellular immune response towards a purified protein of the PreS1-S2-S region of HBV (0.25–1.0 μg/ml, adw subtype, kindly provided by Dr. K.-H. Heermann, Göttingen, Germany) was determined in 52 booster vaccinated probands. In contrast to standardized LTT against 12 recall antigens, this assay used 200 000 PBMCs per culture.

2.4. Chemotaxis assay

PBMCs were resuspended in RPMI 1640 plus 1% fatty acid-free bovine serum albumin (BSA) (Sigma, Deisenhofen, Germany) and transferred to cell culture dishes. After 30 min the non-adherent cells were collected, centrifuged and resuspended in RPMI 1640 plus 1% fatty acid-free BSA. All cell preparations consisted of at least 95% mononuclear cells, as determined by staining with Diff-Quick® (Dade Behring AG, Marburg, Germany), and the fraction of CD3$^+$ T lymphocytes was determined to be $>90\%$ by flow cytometry (CD3 monoclonal antibody (mAb), Becton Dickinson, Heidelberg, Germany). Peripheral blood lymphocytes (PBLs) were resuspended in RPMI 1640 plus 10% heat-inactivated fetal calf serum, 1 μg/ml PHA and 100 U/ml IL-2 for activation and left in a humidified atmosphere with 5% CO_2 at 37°C for 72 h. CD4$^+$ and CD8$^+$ T lymphocytes were prepared by negative selection with Cellect™ cell enrichment immunocolumns (Cytovax Biotechnologies, Edmonton, Canada). In some experiments the cells were pretreated for 24 h with 100 ng/ml PTX. Chemotaxis of PBLs and subsets of T lymphocytes was determined in a 48-well microchemotaxis chamber as described [2] with minor modifications: stromal cell-derived factor 1α (SDF-1α, Peprotech, Rocky Hill, USA) was diluted in RPMI 1640 with 0.1% fatty acid-free BSA. The PBL suspension was adjusted to a density of 2×10^6 cells/ml RPMI 1640 with 1% fatty acid-free BSA. 50 μl of the cell suspension was applied to the upper wells of the microchemotaxis chamber separated from the lower wells by a polyvinylpyrrolidone-free polycarbonate filter membrane with a pore size of 5 μm coated overnight with 6.7 μg/ml fibronectin (Sigma). Migration of PBLs was allowed for 2 h in a humidified atmosphere with 5% CO_2 at 37°C. Migrated cells were quantified on the lower side of the filter after staining with Diff-Quick® by counting cells in three random areas of each well under a microscope with a magnification of 400-fold (high power field, HPF) and calculating the mean per HPF.

2.5. Flow cytometric analysis

Three-part differentiation of PBMCs was performed with lysed whole-blood by flow cytometry (Calibur, Becton Dickinson, Heidelberg, Germany) using CD14 and CD45 mAb. Lymphocyte subsets were identified with the mAb combinations: CD3/CD4/CD8, CD3/CD19, CD3/CD25, CD4/CD49d (integrin VLA4), CD4/CD62L (L-selectin), and CD3/HLA-DR. Analysis of CD4$^+$ or HLA-DR$^+$ T cells was conducted additionally using a linear format to measure channel fluorescence intensities as numerals and to calculate mean fluorescence intensity (MFI) values [5].

2.6. Statistical analysis

Unpaired, two-tailed Student's *t*-test was used to compare lymphocyte proliferation in response to recall antigens and IL-2 as well as chemotaxis between the TT, TC, and CC genotype groups. Cell counts were compared using two-tailed Kruskal–Wallis or Wilcoxon tests. Differences were regarded statistically significant at $P<0.05$.

3. Results

3.1. Enhanced in vitro response of lymphocytes from 825T allele carriers to recall antigens and IL-2, but not to mitogens

The proliferative response of PBMCs obtained from individuals with TT ($n=14$), TC ($n=22$), and CC ($n=27$) genotypes following stimulation with four common recall antigen is summarized in Fig. 1A–D. We observed a 2–4-fold increased proliferation of T cells from individuals homozygous for the 825T allele compared to homozygous C825 allele carriers. This increase reached statistical significance ($P<0.05$) for the following antigens tested: tuberculin (PPD): $66\,240\pm11\,160$ vs. $27\,220\pm5029$, tetanus: $86\,670\pm17\,870$ vs. $38\,610\pm7545$, *Candida albicans*: $45\,190\pm7667$ vs. $26\,290\pm3809$, cytomegalovirus: $13\,480\pm4444$ vs. 5291 ± 1672, herpes simplex virus (HSV)-type 1: $32\,750\pm8561$ vs. $11\,160\pm2467$, Mumps: $11\,070\pm3568$ vs. 2975 ± 514, influenza-A: $14\,430\pm5118$ vs. 6397 ± 1022, and influenza-B: $14\,380\pm4636$ vs. 3936 ± 759 (all values are means of cpm increment of the second highest value ± standard error of the mean (S.E.M.)). Responses towards antigens like diphtheria, rubella, *Varicella zoster* virus, and measles showed a similar tendency which just escaped statistical significance (data not shown). In addition, antigen-induced cell proliferation was enhanced in individuals with TT vs. TC genotype and reached statistical significance for PPD and HSV-type 1 ($P<0.05$). Furthermore, the proliferative response to IL-2 (25 and 100 U/ml) was also significantly ($P<0.05$) increased in cells of homozygous T allele vs. homozygous C allele blood donors (Fig. 1E). After IL-2 stimulation (25 U/ml) increments were $85\,090\pm21\,840$ cpm ($n=9$), $61\,680\pm17\,450$ cpm ($n=10$), and $36\,990\pm7119$ cpm ($n=16$) of individuals with TT, TC, and CC genotypes, respectively. The above results persisted after correction for increased CD4$^+$ cell counts in 825T allele carriers. In contrast, after stimulation with mitogens the proliferative response of PBMCs was not significantly different between genotypes. Mean cpm and S.E.M. values for TT vs. CC allele carriers were $198\,200\pm17\,430$ vs. $225\,400\pm12\,600$ (PHA), $133\,300\pm10\,880$ vs. $161\,800\pm7452$ (concanavalin A), $59\,630\pm8869$ vs. $60\,880\pm5737$ (pokeweed mitogen), and $102\,100\pm15\,420$ vs. $100\,500\pm9991$ (OKT3).

Antigen- and IL-2-induced proliferations were actually dependent on activation of PTX-sensitive Gi proteins as confirmed by experiments in which lymphocytes of 23 individuals were pretreated with 100 ng/ml PTX for 24 h prior to stimulation. T lymphocyte proliferation against recall antigens was significantly ($P<0.05$) reduced to $28\pm14\%$ (range for the different antigens 8–66%), against mitogens to and against IL-2 to $58\pm8\%$ of controls. This inhibition by PTX was independent of C825T allele status (data not shown).

Expanding the data to a more representative group of 52 booster-vaccinated probands (TT: $n=4$, TC: $n=20$, CC: $n=28$) cellular in vitro responses against PreS1-S2-S HBV antigen showed a significant ($P<0.05$) 2.5-fold increase in PBMCs from individuals carrying the 825T allele compared to those homozygous for the C825 allele (Table 1). In this setting TT and TC carriers were analyzed together because of the small size of the TT group; the cpm increment values were $31\,230\pm25\,620$ (TT) and $32\,460\pm6836$ (TC).

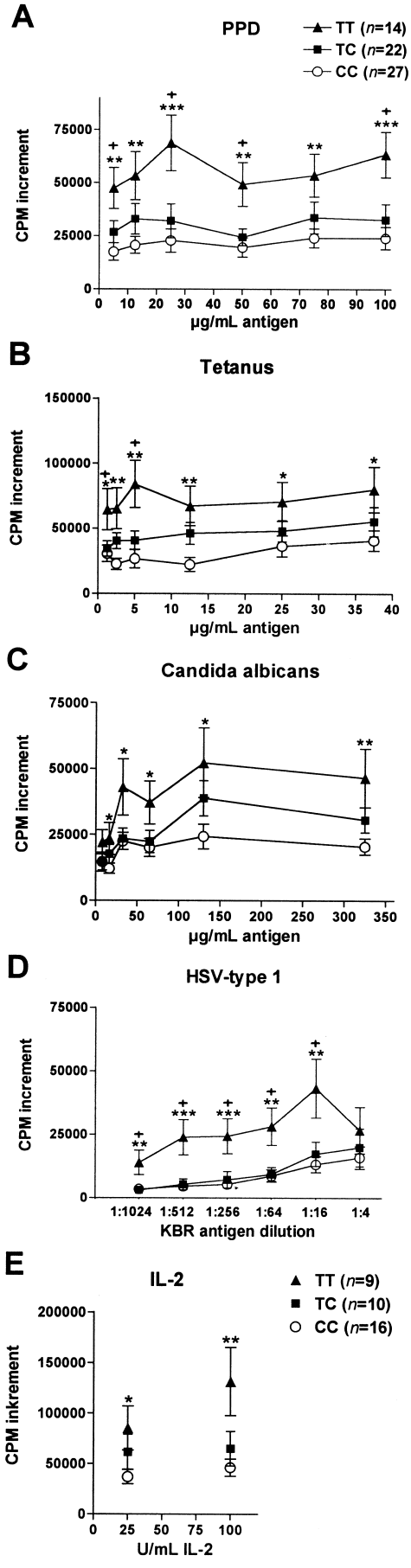

Fig. 1. TT, TC, and CC *GNB3* genotypes and T cell responses. LTT towards common recall antigens (A–D) and IL-2 (E) showed 2–4-fold increases in cell proliferation comparing TT and CC probands. Data are presented for PPD, tetanus, *C. albicans*, and HSV-type 1, the most widely distributed antigens tested. Data are given as mean ± S.E.M. Unpaired Student's *t*-tests were performed separately for each antigen/IL-2 concentration. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for TT vs. CC genotypes; $^{+}P < 0.05$ for TT vs. TC genotypes.
←

3.2. Enhanced chemotactic response of lymphocytes

Stimulation of non-activated PBLs with SDF-1α, a chemokine which activates T cells via the G protein-coupled CXCR4 receptor [6,7], induced chemotaxis at concentrations above 10 ng/ml with maximum efficacy at 1000 ng/ml (Fig. 2A). The chemotactic response of lymphocytes from 825T allele carriers was significantly enhanced ($P < 0.05$) compared to that from homozygous C825 allele carriers (Fig. 2A). After activation of cells for 72 h with PHA and IL-2 this effect was even more pronounced (Fig. 2B). The efficacy and potency of SDF-1α to induce migration of lymphocytes were increased with an optimum at 300 ng/ml. Significantly enhanced chemotaxis of lymphocytes from homo- or heterozygous 825T allele carriers was observed at 10–300 ng/ml SDF-1α. Enrichment of $CD4^+$ and $CD8^+$ T cells by negative selection suggested that the enhanced response is apparently restricted to $CD4^+$ T lymphocytes (Fig. 2C). The total number of migrated cells was significantly different at 1000 ng/ml SDF-1α: 247.8 ± 44.1 (TT+TC genotype) vs. 88.7 ± 13.9 cells per HPF. In contrast, SDF-1α-stimulated chemotaxis of $CD8^+$ T lymphocytes was independent of C825T allele status. PTX completely abolished the SDF-1α-induced chemotaxis of all T lymphocytes indicating the involvement of Gi proteins in CXCR4 signaling (data not shown).

3.3. Differences in leukocyte subsets

825T allele (TT+TC) carriers had significantly higher $CD3^+CD4^+$ counts ($n = 100$, mean ± S.E.M.: 43.4 ± 0.7%, 778 ± 26 cells/µl) than homozygous C825 allele carriers ($n = 102$, 40.7 ± 0.7%, 694 ± 20 cells/µl). However, there was no difference between individuals homo- and heterozygous for the T allele (TT: $n = 17$, 42.6 ± 1.9%, 761 ± 55 cells/µl; TC: $n = 83$, 43.6 ± 0.7%, 782 ± 29 cells/µl). In contrast, we could not detect significant differences for $CD3^+CD8^+$, $CD19^+$, or $CD14^+$ cell counts between genotypes. Markers of cell activation and adhesion such as CD25 and HLA-DR on T cells, and CD49d (integrin VLA4) and CD62L (L-selectin) on $CD4^+$ T cells did not differ between carriers and non-carriers of the 825T allele (TT+TC: $n = 20$, CC: $n = 10$). Measuring MFI of CD4 and HLA-DR expression on T cells did also not yield any difference (TT+TC: $n = 20$, CC: $n = 10$).

Table 1
Cellular in vitro response towards HBV antigen after HBV booster

GNB3 genotype		Increment (cpm)
TT+TC	($n = 24$)	32 250 ± 6 815[a]
CC	($n = 28$)	13 030 ± 5 347
Total	($n = 52$)	21 900 ± 4 429

LTT towards purified PreS1-S2-S HBV antigen in 825T allele carriers showed a 2.5-fold increase in cell proliferation using 2×10^5 PBMCs. Data are given as mean ± S.E.M.
[a] $P < 0.05$ for TT+TC vs. CC genotypes.

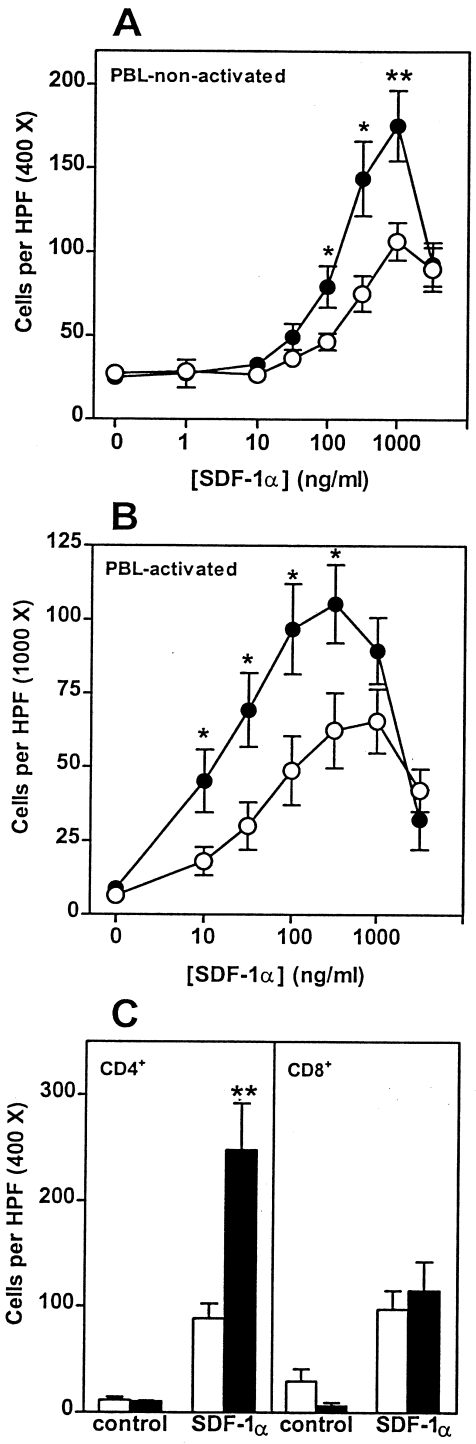

Fig. 2. *GNB3* genotypes and T cell chemotaxis. (A) Migration of PBLs was stimulated with the indicated concentrations of SDF-1α for 2 h in a 48-well microchemotaxis chamber. Open circles, CC genotype ($n=14$); filled circles, TC ($n=11$) and TT ($n=5$) genotypes. (B) Chemotactic response of PHA/IL-2-activated PBLs in response to the indicated concentrations of SDF-1α: open circles, CC genotype ($n=11$); filled circles, TC ($n=10$) and TT ($n=1$) genotypes. (C) Chemotaxis of negatively selected $CD4^+$ or $CD8^+$ in response to stimulation with 1000 ng/ml SDF-1α: open bars, CC genotype ($n=5$); filled bars, TC ($n=4$) and TT ($n=1$) genotypes. Data represent the mean ± S.E.M. of migrated cells per HPF. $*P<0.05$ and $**P<0.01$ for the difference between TT+TC vs. CC genotypes.

4. Discussion

The *GNB3* polymorphism clearly defines different levels of T lymphocyte response capacity as proliferation was significantly elevated in TT vs. CC allele carriers after stimulation with common recall antigens. The in vitro antigen stimulation mimics the in vivo situation of a second or repeated contact with a specific antigen. Specific cellular recognition of the antigens tested in vitro could be either caused by preceding infections, e.g. with *C. albicans* or by vaccinations, e.g. against tetanus, diphtheria, or HBV. As could be demonstrated in booster vaccinated probands cellular immune response following a repeated in vivo contact with an HBV antigen resulted in a more vigorous in vitro response in individuals carrying the 825T allele. Therefore, one may assume that vaccinations in 825T allele carriers could be more effective, i.e. less doses of vaccine or longer intervals between vaccinations may be feasible to achieve comparable cellular responses. In addition, it could be postulated that 825T allele carriers may better defeat infectious diseases in which cellular activation involving T cells is essential. The impact of *GNB3* genotypes on infectious and inflammatory diseases has been mentioned in kidney allograft recipients [8]. It was described that these diseases when leading to death were significantly less frequent in recipients with 825T graft.

Apart from *GNB3* also the polymorphic HLA system controls cellular and humoral immune responses [9,10]. HLA molecules are required for antigen-derived peptide presentation and activation of T lymphocytes and play a central role in the specific immune response. Especially after HBV-specific vaccination an effect of certain HLA class II alleles on the rate of good or poor antibody response was described [10]. In terms of immune response an interaction between HLA and *GNB3* genes located on chromosomes 6p and 12p, respectively, should not be expected.

PTX inhibition experiments confirmed that Gi proteins are involved in T cell signal transduction following stimulation with recall antigens and IL-2 [11]. Data on T cells and the involvement of a G protein in the antigen, and cytokine-induced responses are still controversial. Some evidence indicated that a G protein couples the T cell receptor to phospholipase C [12]. But direct evidence is lacking supporting the involvement of a G protein in T cell receptor-mediated activation of phospholipase C-γ1 [13]. Other authors refused the idea of this direct coupling [14]. In contrast, Gi protein involvement in the antigen receptor-mediated signal transduction has been clearly shown for B lymphocytes [15]. It was described that a PTX-sensitive G protein regulates tyrosine phosphorylation of phospholipase C-γ1 by controlling the activity of a protein tyrosine kinase. Following these data, one can assume that T cells of *GNB3* 825T carriers display an enhanced G protein activation after stimulation with recall antigens and IL-2 (but not with mitogens), resulting e.g. in a longer or more effective activation of phospholipase C or an intermediate molecule via Gβ3-s.

Besides, the chemotactic response of T lymphocytes, especially $CD4^+$ T cells from 825T allele carriers, was significantly enhanced compared to homozygous C825 allele carriers. These findings are unlikely fortuitous for several reasons: (i) chemotaxis is known to be regulated via Gβγ subunits released from PTX-sensitive G proteins [16,17], (ii) increased chemotaxis was recently found enhanced in neutrophils from

GNB3 825T allele carriers [2], and (iii) the transfection of the splice variant Gβ3-s associated with the 825T allele into COS-7 cells causes enhanced neutrophil chemotaxis [3]. In contrast to antigen-specific cell proliferation, heterozygosity for the 825T allele was sufficient for this effect and no further enhancement was observed in cells from homozygous 825T allele carriers. As the *GNB3* C825T allele status obviously influences chemotaxis, antigen-specific cellular immune response as well as IL-2-induced proliferation, there seems to exist one common central step of signal transduction in $CD4^+$ T cells that may be facilitated in cells expressing Gβ3-s associated with the 825T allele.

$CD4^+$ counts were significantly elevated in TT+TC vs. CC carriers. As $CD4^+$ cells are essential for specific cellular immune response, their increased numerical presence should result in an enhanced immune function. In contrast to $CD4^+$ the $CD8^+$ cell counts were not influenced by the *GNB3* polymorphism. Very recently it was shown that the G protein-coupled receptors GPK2 and Edg-4 are differently expressed on $CD4^+$ cells compared to $CD8^+$ cells [18,19]. It is therefore conceivable that predominantly $CD4^+$ T cells are influenced by the G protein polymorphism *GNB3*. Few data are available on the regulation of $CD4^+$ cell counts in humans: family analysis showed a genetic control of the CD4/CD8 T cell ratio in humans through yet undefined genes [20]. This ratio also appears to be influenced by race and environmental factors [21,22]. The expression of the CD4 gene is controlled by at least five distinct transcription control elements, including the promoter, three enhancers, and a silencer designated HES (hairy/enhancer of split homologue)-1 [23]. The exact mechanisms underlying increased $CD4^+$ cell counts in 825T allele carriers remain to be defined. We have no evidence for an increased production of $CD4^+$ cells and, thereby, total mass of $CD4^+$ cells. Increased chemotactic response to certain cytokines could be one decisive factor. On the other hand, increased counts of $CD4^+$ cells in 825T allele carriers could result from an altered distribution of $CD4^+$ cells between lymphatic organs and peripheral blood. In mice, thymocyte emigration and migration of B and T lymphocytes into splenic white pulp cords is PTX-sensitive and, thus, involves Gi proteins [24,25]. Another possible mechanism explaining higher $CD4^+$ counts in 825T allele carriers could be a decrease in apoptosis – this has to be further analyzed.

Our results described above may help to understand and to predict individual cellular immune reactions, e.g. in the cause of HIV-1 or microbial infections, allotransplantation, or autoimmune phenomena. As could be shown recently, kidney allograft survival is decreased in grafts from donors homozygous for the 825T allele [8]. Perpetuated arteriolar media hypertrophy which leads to progressive allograft injury was supposed as mechanism occurring more frequent in homozygous 825T allele carriers. It is likely that other immune functions and disorders are as well influenced by C825T allele status. The data presented here may help to elucidate the extent and contribution of altered cellular immune functions in homo- and heterozygous 825T allele carriers associated with different disease outcomes.

Acknowledgements: We thank Dr. K.-H. Heermann, Abteilung für Medizinische Mikrobiologie, Georg-August-Universität Göttingen, Germany, for the PreS1-S2-S HBV antigen and M. Huben, B. Nyadu, G. Siffert, S. Wortmann, and K. Zelinski for their excellent technical assistance. This article is a partial fulfilment of requirements for the doctor's degree at the Medical Faculty, University of Essen, for Mr. F. Ramann and Mr. W. Barsegian. The study was partly supported by the Ministerium für Schule und Weiterbildung, Wissenschaft und Forschung (MSWWF) des Landes Nordrhein-Westfalen and by the IFORES program of the University Hospital of Essen, Medical School.

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