

Article

GTS-21 Enhances Regulatory T Cell Development from T Cell Receptor-activated Human CD4⁺ T Cells Exhibiting Varied Levels of *CHRNA7* and *CHRFAM7A* Expression

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Abstract: Immune cells such as T cells and macrophages express $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7$ nAChRs), which contribute to the regulation of immune and inflammatory responses. Earlier findings suggest $\alpha 7$ nAChR activation promotes the development of regulatory T cells (Tregs) in mice. Using human CD4⁺ T cells, we investigated the mRNA expression of the $\alpha 7$ subunit and the human-specific dup $\alpha 7$ nAChR subunit, which functions as a dominant-negative regulator of ion channel function, under resting conditions and T cell receptor (TCR)-activation. We then explored the effects of the selective $\alpha 7$ nAChR agonist GTS-21 on proliferation of TCR-activated T cells and Treg development. Varied levels of mRNA for both the $\alpha 7$ and dup $\alpha 7$ nAChR subunits were detected in resting human CD4⁺ T cells. mRNA expression of the $\alpha 7$ nAChR subunit was profoundly suppressed on days 4 and 7 of TCR-activation as compared to day 1, whereas mRNA expression of the dup $\alpha 7$ nAChR subunit remained nearly constant. GTS-21 did not alter CD4⁺ T cell proliferation but significantly promoted Treg development. These results suggest the potential *ex vivo* utility of GTS-21 for preparing Tregs for adoptive immunotherapy, even with high expression of the dup $\alpha 7$ subunit.

Keywords: acetylcholine; adoptive immunotherapy; $\alpha 7$; dup $\alpha 7$; GTS-21; nAChR; Treg

1. Introduction

Immune cells such as T cells, B cells and monocytes express various subtypes of both muscarinic and nicotinic acetylcholine (ACh) receptors (mAChRs and nAChRs, respectively) [1-5]. Moreover, they also express mRNA for ACh synthase (choline acetyltransferase, ChAT) and synthesize ACh [1-11]. Activation of T-cell receptors (TCRs) by phytohemagglutinin (PHA) or anti-CD3/CD28 monoclonal antibodies or by activation of protein kinases A and C, enhances ChAT mRNA expression and increases ACh synthesis and release [8,12]. These findings indicate that ACh synthesized by immune cells acts in an autocrine and paracrine manner via AChRs on immune cells, especially T cells, and is involved in regulating immune function.

Among the various mAChRs and nAChRs subtypes expressed by immune cells, $\alpha 7$ nAChR has received much attention. This is because $\alpha 7$ nAChR activation in lipopolysaccharide (LPS)-treated mice prevents septic shock by inhibiting the synthesis and release of the pro-inflammatory cytokine

tumor necrosis factor- α (TNF- α) from macrophages [13]. In addition, $\alpha 7$ nAChR gene-deficient ($\alpha 7$ -KO) mice immunized with ovalbumin had higher serum concentrations of anti-ovalbumin-specific IgG₁ than identically treated wild-type mice. At the same time, synthesis of the pro-inflammatory cytokines TNF- α , interferon- γ (IFN- γ) and IL-6 was up-regulated in spleen cells from ovalbumin-immunized $\alpha 7$ -KO mice [14]. On the other hand, ACh produced in T cells and $\alpha 7$ nAChRs expressed on macrophages are known to play key roles in various cholinergic anti-inflammatory pathways [15-20]. Together, these findings indicate the involvement of $\alpha 7$ nAChRs on immune cells in the regulation of inflammatory and immune functions.

Recently, Mashimo et al (2019) found that GTS-21, a partial $\alpha 7$ nAChR agonist [21], promotes the differentiation of TCR-activated mouse CD4⁺ T cells into regulatory T cells (Tregs) and effector helper T (Th1, Th2, and Th17) cells [22]. However, GTS-21 suppresses antigen-processing and antigen-presenting cell (APC)-dependent activation of mouse CD4⁺ T cell differentiation. This suggests that $\alpha 7$ nAChRs play a variety of roles affecting immune function, and that their effects depending on the cells in which they are expressed (CD4⁺ T cells or APCs) [22,23].

The gene encoding the human neuronal $\alpha 7$ nAChR subunit *CHRNA7* is located on chromosome 15 and contains 10 exons. Exons 1-6 encode the extracellular N-terminal region of the receptor, including the ligand-binding domain, and exons 7-10 encode the channel region [24]. Notably, chromosome 15 also contains a human-specific partial duplicate $\alpha 7$ nAChR subunit-like gene with exons 5-10. This gene rearranges with the kinase gene *FAM7A* on chromosome 3 to form a hybrid, *CHRFAM7A* [25]. The *CHRFAM7A* gene product, dup $\alpha 7$, lacks a ligand-binding region and assembles with intact $\alpha 7$ subunits to form $\alpha 7$ nAChRs composed of a total of five $\alpha 7$ and dup $\alpha 7$ subunits in various ratios [26]. Because dup $\alpha 7$ acts as a dominant negative regulator of ion channel function, $\alpha 7$ nAChRs with a large dup $\alpha 7$ component do not function well as ion channels, despite retaining of channel structure [26-31]. Neuronally expressed $\alpha 7$ nAChRs, which function mainly as ligand-gated ion channels play key roles in cellular signaling, and their dysfunction due to widespread expression of dup $\alpha 7$ is thought to be associated with several central nervous system disorders, including schizophrenia and certain forms of cognitive deficits (see reviews by Bertrand et al. (2015) and Bertrand and Terry (2017)) [32,33]. Multiple clinical trials with $\alpha 7$ nAChR agonists failed to demonstrate efficacy in patients with cognitive deficits or schizophrenia, suggesting decreased expression of functional $\alpha 7$ nAChRs in these patients' neuronal cells [32,33]. However, most $\alpha 7$ nAChR agonists, including GTS-21, have been shown clinically to be safe [32,34].

Evidence now suggests that $\alpha 7$ nAChRs have dual functions as canonical ionotropic channels and as non-canonical metabolic signaling receptors in both neuronal and non-neuronal cells [35]. $\alpha 7$ nAChRs with metabotropic function are coupled to heterotrimeric G proteins such as G α_q and activate a cascade of signals leading to the release of Ca²⁺ from intracellular stores [35-38]. In immune cells, $\alpha 7$ nAChRs appear to function as metabotropic receptors rather than as ionotropic receptors [22,39-41]. The functional effects of dup $\alpha 7$ contained within metabotropic $\alpha 7$ nAChRs are not yet known. Considering the potential utility of $\alpha 7$ nAChR agonists as immunomodulatory agents [13,14,19,22,23], it is noteworthy that human peripheral blood leukocytes express more *CHRFAM7A* than *CHRNA7* [31,42,43]. We therefore investigated the mRNA expression of both $\alpha 7$ and dup $\alpha 7$ subunits in human CD4⁺ T cells and the effect of GTS-21 on Treg development.

2. Results

2.1. mRNA expression of $\alpha 7$ and dup $\alpha 7$ subunits

2.1.1. Under the resting conditions

Figure 1 shows a scatterplot of *CHRNA7* and *CHRFAM7A* expression for each individual divided by the values closest to their respective medians to compare the magnitude of interindividual variability in *CHRNA7* and *CHRFAM7A* expression. Varied expression of both *CHRNA7* and *CHRFAM7A* were detected in all cryopreserved human peripheral blood CD4⁺ T cells examined under the resting conditions. Interindividual variation was greater for *CHRNA7* expression than for *CHRFAM7A* expression (note the log scale of the vertical axis).

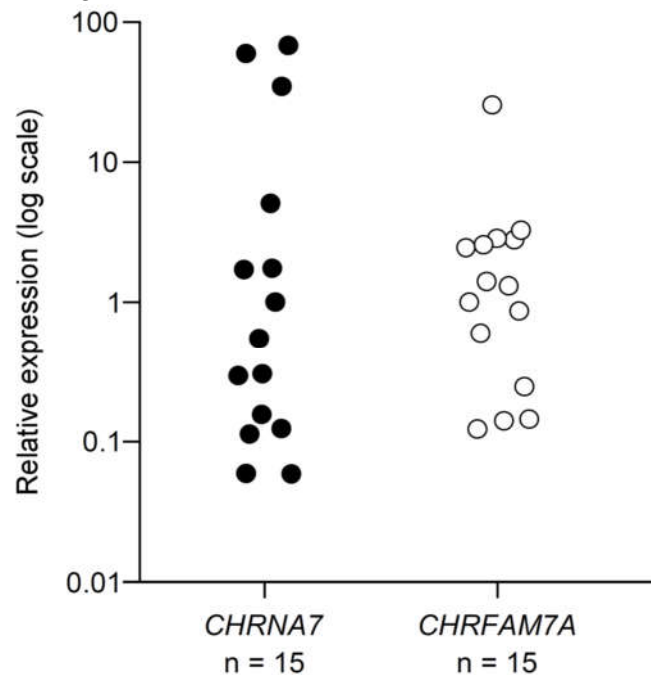


Figure 1. Expression of $\alpha 7$ and dup $\alpha 7$ subunit mRNAs in resting human CD4⁺ T cells. *CHRNA7* and *CHRFAM7A* ($\alpha 7$ and dup $\alpha 7$ subunit mRNA, respectively) expression levels were first normalized to *GAPDH* mRNA in each individual. Then to compare the magnitude of interindividual variability in *CHRNA7* and *CHRFAM7A* expression, levels of *CHRNA7* and *CHRFAM7A* mRNA were divided by the values closest to their respective medians and plotted on a logarithmic scale. The interindividual variability of *CHRNA7* expression was statistically greater than that of *CHRFAM7A* expression ($F_{(14,14)} = 2.18 \times 10^{-5}$). The difference between the standard deviations was assessed with the F-test.

2.1.2. Changes during TCR-activation

Expression of *CHRNA7* in cells cultured in RPMI medium containing 20 $\mu\text{g/ml}$ IL-2 (Control) was nearly unchanged over the 7-day observation period (Figure 2A). TCR activation with Human T-activator CD3/CD28 Dynabeads did not alter *CHRNA7* expression on day 1, but the expression levels were significantly suppressed on days 4 and 7. Addition of 30 μM GTS-21 to the culture did not affect *CHRNA7* expression in the TCR-activated group over the 7-day observation period.

Under control conditions, levels of *CHRFAM7A* expression did not fluctuate over the 7-day observation period (Figure 2B). Moreover, TCR activation in the absence or presence of 30 μM GTS-21 did not affect *CHRFAM7A* expression at any time during the 7-day observation period.

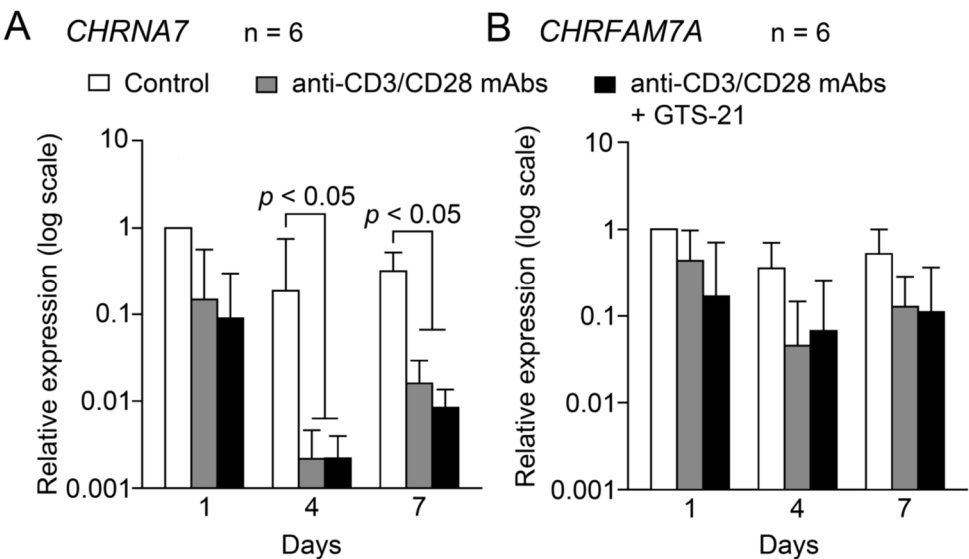


Figure 2. Fluctuations in mRNA expression of the $\alpha 7$ and dup $\alpha 7$ subunits during TCR activation. Human CD4⁺ T cells were cultured for up to 7 days in the standard culture medium in the presence or absence of Human T-activator CD3/CD28 Dynabeads at a beads-to-cell ratio of 1:1 with or without 30 μ M GTS-21. Levels of *CHRNA7* and *CHRFAM7A* expression in cells from each individual was first normalized to *GAPDH* expression. Then to detect fluctuations over time induced by TCR activation, *CHRNA7* (A) and *CHRFAM7A* (B) mRNA levels were further normalized to the respective levels in controls observed on day 1. Bars are the geomean \pm S.E.M. ($n = 6$). Statistical significance was assessed with two-way ANOVA and *post-hoc* Tukey tests.

2.2. Effects of GTS-21 on CD4⁺ T cell proliferation and Treg development

2.2.1. Proliferation

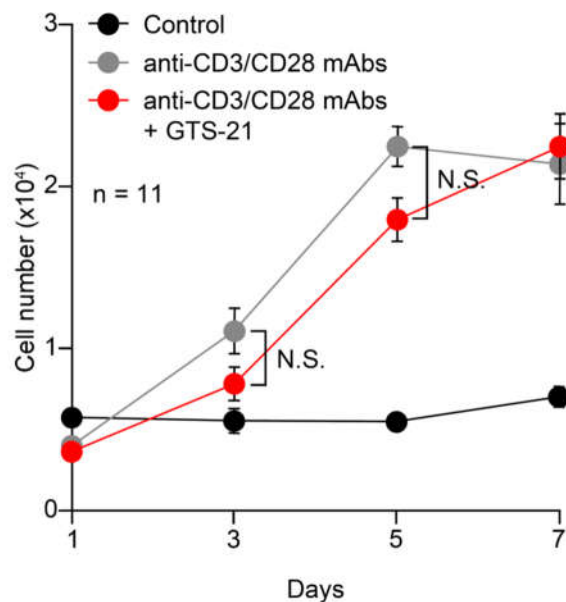


Figure 3. Effects of GTS-21 on CD4⁺ T cell proliferation. Human CD4⁺ T cells were cultured for up to 7 days in the standard medium in the presence of Human T-activator CD3/CD28 Dynabeads at a beads-to-cell ratio of 1:1 with or without 30 μ M GTS-21. Cell numbers were counted after staining with trypan blue. Data are shown as the mean \pm S.E.M. ($n = 11$). N.S.; not significant (two-way ANOVA with *post-hoc* Tukey test).

Numbers of CD4⁺ T cells remained nearly constant over the 7-day culture period in RPMI medium containing 20 ng/ml IL-2 (Figure 3). With TCR activation, however, CD4⁺ T cell proliferation was significantly enhanced on culture days 5 and 7. Addition of 30 μ M GTS-21 caused no further enhancement of proliferation compared to TCR activation alone.

2.2.2. Treg development

Figure 4A shows flow cytometric plots for CD4⁺CD25⁺FoxP3⁺ T cells (Tregs) from a representative individual in the absence and presence of 30 μ M GTS-21 on day 5 of culture. The data show that GTS-21 promoted Treg development from CD4⁺ T cells.

GTS-21 promoted Treg development to varying degrees in samples from all individuals on day 5 of culture in the presence of 20 ng/ml IL-2 and 5 ng/ml transforming growth factor- β (TGF- β). The average number of Tregs was significantly higher in cultures with GTS-21 than without it ($p = 0.008$) (Figure 4B).

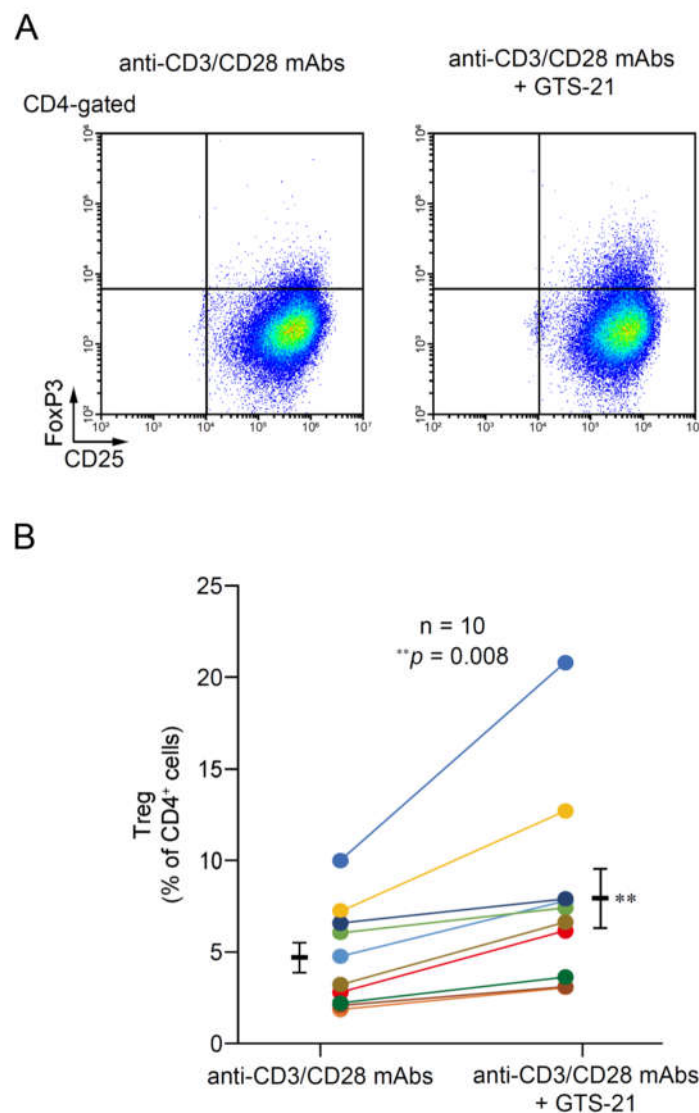


Figure 4. Effects of GTS-21 on Treg development. (A) Representative flow cytometric plots for Treg development among TCR-activated human CD4⁺ T cells in the presence or absence of 30 μ M GTS-21. (B) GTS-21 enhanced Treg development from TCR-activated human CD4⁺ T cells on day 5 of culture. Gates were used to calculate the percentages of CD4⁺CD25⁺FoxP3⁺ cells. For comparison, a line connects the percentage of Tregs observed in the absence and presence of 30 μ M GTS-21 among cells from the same individuals. Bars are means \pm S.E.M. (n = 10). Statistical significance was assessed using paired *t*-tests (** $p < 0.01$).

3. Discussion

3.1. Expression of mRNAs for $\alpha 7$ and dup $\alpha 7$ subunits in resting human CD4⁺ T cells

Varied levels of both $\alpha 7$ and dup $\alpha 7$ subunits mRNAs were detected in cryopreserved CD4⁺ T cells from normal volunteers. However, it is not yet clear whether the large interindividual variation in $\alpha 7$ and dup $\alpha 7$ subunit expression is due to genetic disposition, immunological regulation or both. The greater interindividual variation in $\alpha 7$ subunit expression suggests $\alpha 7$ nAChRs in CD4⁺ T cells are likely more susceptible to immune stimulation in daily life. Expression of $\alpha 7$ and dup $\alpha 7$ subunit mRNAs showed no clear trends across gender, age or ethnicity.

3.2. Expression of $\alpha 7$ and dup $\alpha 7$ subunit mRNAs during TCR activation

Changes in the mRNA expression of the $\alpha 7$ and dup $\alpha 7$ subunits during TCR activation were explored for the first time and provide valuable information about the dynamics of $\alpha 7$ nAChRs in human CD4⁺ T cells. Activation of TCRs on T cells up-regulates ChAT mRNA expression and ACh synthesis [8-10,12,44-46]. PHA induces ChAT mRNA expression in human mononuclear cells; induction is first evident after 48 h of exposure, and the effect persists for at least 72 h [8]. PHA-activated MOLT-3 cells, a human leukemic T cell line, secrete sufficient amounts of ACh to activate AChRs in an autocrine and paracrine manner [47]. It is therefore suggested that sustained exposure to elevated concentrations of ACh released from activated T cells attenuates *CHRNA7* expression. Indeed, the plasticity of *CHRNA7* expression has been confirmed in TCR-activated T cells [46-48]. The marked down-regulation of *CHRNA7* expression in TCR-activated CD4⁺ T cells after 4 days in culture observed in the present study can be attributed to negative regulation of *CHRNA7* expression mediated by sustained $\alpha 7$ nAChR activation induced by ACh synthesized and released from CD4⁺ T cells. This in turn suggests $\alpha 7$ nAChRs are involved in regulating CD4⁺ T cell function. The absence of significant variation in *CHRFAM7A* expression during TCR activation indicates dup $\alpha 7$ nAChRs are less involved in ACh-mediated regulation of CD4⁺ T cell function.

In contrast to the dynamic fluctuation in *CHRNA7* expression, the nearly constant *CHRFAM7A* expression in TCR-activated CD4⁺ T cells is consistent with the finding that *CHRNA7* and *CHRFAM7A* are independently regulated by their respective promoters driving their differential expression [31,42,49]. Our finding that GTS-21 did not affect *CHRFAM7A* expression in human CD4⁺ T cells differed from that in human monocyte-derived macrophages, where nicotine suppresses *CHRFAM7A* expression [26]. This suggests that regulation of *CHRFAM7A* is cell type-specific and that dup $\alpha 7$ nAChRs are less involved in ACh-mediated regulation of CD4⁺ T cell function.

Recent studies revealed that the $\alpha 7$ nAChRs have both ionotropic and metabotropic functions in neurons [36,37,50]. Moreover, most studies indicate that $\alpha 7$ nAChRs in macrophages and T cells function as metabotropic receptors independent of ionotropic signaling [23,39-41,51-54]. In oocytes injected with various combinations of dup $\alpha 7$ and $\alpha 7$ mRNAs, decreased ionotropic function of $\alpha 7$ nAChRs was observed with increasing dup $\alpha 7$ / $\alpha 7$ composition ratios [26]. Under the present experimental conditions, $\alpha 7$ nAChRs in TCR-activated CD4⁺ T cells may have elevated dup $\alpha 7$ / $\alpha 7$ subunit ratios after 4 days in culture and little ionotropic function. There is currently little evidence to suggest that the dup $\alpha 7$ subunit plays a specific physiological role in T cells, and the present findings indicate there is little if any involvement of ionotropic signaling in the regulation of CD4⁺ T cell function.

3.3. Effects of GTS-21 on proliferation

In J774A.1 cells (a mouse macrophage cell line), GTS-21 mitigates LPS-induced proliferation arrest for up to 9 h [55]. In mice, proliferation of activated T cells is reportedly inhibited by stimulation of a $\alpha 7$ nAChR-mediated pathway [56]. In the present study, however, GTS-21 did not affect the proliferation of highly purified TCR-activated human CD4⁺ T cells. It is unclear why $\alpha 7$ nAChR agonists have different effects on mouse and human T cell proliferation; little information is currently available on the effects of $\alpha 7$ nAChR agonists, including GTS-21, on human T cell proliferation.

3.4. The effect of GTS-21 on Treg development in TCR-activated CD4⁺ T cells

As in our earlier study of TCR-activated CD4⁺ T cells from mice [22], GTS-21 (30 μ M) promoted Treg development from human TCR-activated CD4⁺ T cells, even in the presence of appreciable levels of *CHRFAM7A* expression, thereby increasing the proportion of Tregs to about 1.8-fold over the control in 5 days. This suggests *ex vivo* utilization of GTS-21 could potentially help to reduce the time required to prepare sufficient numbers of Tregs for adoptive immunotherapy and reduce the associated costs. It remains possible to continue to improve Treg development through further optimization of culture conditions, including the GTS-21 concentration, beads-to-cells ratio (currently 1:1) and culture duration.

An earlier study [26] indicated that under conditions where *CHRNA7* expression is profoundly suppressed, as observed in TCR-activated CD4⁺ T cells on days 4 and 7 of the present experiment, most of the subunits that make up $\alpha 7$ nAChRs may be occupied by dup $\alpha 7$ subunits, which lack a ligand-binding site. Nevertheless, GTS-21 promoted Treg development from CD4⁺ T cell (Figure 4). Based on the observation that GTS-21 suppresses TNF- α and IL-6 secretion in LPS-stimulated macrophages from $\alpha 7$ -KO mice, Garg and Loring (2019) reported that GTS-21 may inhibit proinflammatory cytokine production by acting at sites unrelated to $\alpha 7$ nAChRs [57]. However, because Mashimo et al (2019) observed that GTS-21 promotes Treg development from wild-type CD4⁺ T cells but not in $\alpha 7$ -KO mice [22], it seems most likely that GTS-21 promotes Treg development by acting on the $\alpha 7$ subunit of $\alpha 7$ nAChRs. However, to further confirm the involvement of $\alpha 7$ nAChRs in Treg development, the effects of $\alpha 7$ nAChR agonists other than GTS-21 should be assessed.

Metabotropic $\alpha 7$ nAChR function induces the release of G-proteins bound to M3-M4 loop of the channel, activating signaling cascades leading to the release of Ca²⁺ from intracellular stores [58]. The resulting increase in intracellular free Ca²⁺ ion concentration activates protein kinase C, which in turn activates PI3K/Akt signaling pathway, leading to promotion of nuclear translocation of nuclear factor erythroid 2-related factor 2 to the nucleus and overexpression of heme oxygenase-1, and finally resulting in inhibition of pro-inflammatory cytokine production, in macrophages [59,60]. Both $\alpha 7$ and dup $\alpha 7$ subunits retain G-protein binding site in their intracellular M3-M4 loop [35,37,53]. Therefore, the above process should work even if $\alpha 7$ subunit makes up a small fraction of the $\alpha 7$ nAChR. However, it remains unclear how the PI3K/Akt signaling pathway in T cells is involved in promoting Treg development.

TCR activation induces *de novo* IL-2 synthesis and initiates a signaling cascade that leads to activation of Janus kinase 1 (JAK1) and JAK3 [61]. IL-6 signaling is essential for optimal T cell differentiation and is transduced via JAK family proteins, culminating in STAT3 activation [62-64]. STAT3 is a critical positive regulator of T cell differentiation and functions in several CD4⁺ T cell subsets, including Th2 and Th17 cells and Tregs [64-66]. In our earlier study using TCR-activated CD4⁺ T cells from mice, GTS-21 dose-dependently enhanced IL-6 production and significantly promoted Treg development without affecting IL-2 production [22]. In the presence of IL-2 and TGF- β , GTS-21 significantly enhanced Treg development from TCR-activated human CD4⁺ T cells. These findings suggest that enhanced IL-6 production contributes to the up-regulation of Treg development by GTS-21.

In summary, results of the present study demonstrate that TCR-activation of human peripheral blood CD4⁺ T cells expressing variable levels of *CHRNA7* and *CHRFAM7A* profoundly suppressed *CHRNA7* expression with time by day 4 of the experiments, without affecting *CHRFAM7A* expression. GTS-21 did not affect the proliferation induced by TCR-activation. Despite low expression of *CHRNA7* and high expression of *CHRFAM7A*, GTS-21 promoted Treg development in human TCR-activated T cells, increasing the number of Tregs to about 1.8-fold over control in 5 days of culture. In conclusion, these findings support the *ex vivo* utilization of GTS-21 to reduce the time and associated costs of preparing sufficient numbers of Tregs for adoptive immunotherapy.

4. Materials and Methods

4.1. Cell culture

Cryopreserved human peripheral blood CD4⁺ T cells from donors with various backgrounds (15 males and 3 females; 18-80 years old; Ethnicity, 4 African Americans, 4 Asians, 6 Caucasians, 3 Hispanics and 1 mixed ethnicity) (STEMCELL Technologies and Zen-Bio Laboratories) were used for this study. Vials of CD4⁺ T cells were thawed rapidly with vigorous agitation in a 37°C water bath and washed once with RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol and 20 ng/ml IL-2 (the standard medium) at 37°C under a humidified atmosphere with 5% CO₂.

4.1.1. *CHRNA7* and *CHRFAM7A* expression under the resting conditions

Portions of the cells from 15 samples were used for investigation of *CHRNA7* and *CHRFAM7A* expression levels under the resting conditions.

4.1.2. *CHRNA7* and *CHRFAM7A* expression during T cell activation

Cells from six specimens with sufficient cell numbers were used to investigate changes in *CHRNA7* and *CHRFAM7A* expression levels during TCR-activation. The cells (1.5×10^5 cells) were cultured for 7 days in triplicate in a 24-well plate containing 2 ml of the standard medium with or without 30 µM GTS-21. The cells were activated using Human T-activator CD3/CD28 Dynabeads (Veritas, Tokyo, Japan) at a beads-to-cell ratio of 1:1.

4.1.3. Effects of GTS-21 on TCR-activated T cell proliferation

To assess the effect of GTS-21 on cell proliferation, portions of the cells (2×10^4 cells) from 11 samples were cultured for 7 days in duplicate in a 48-well plate containing 200 µl of the standard medium and Human T-activator CD3/CD28 Dynabeads at a beads-to-cell ratio of 1:1. After staining with 4% trypan blue, cell numbers were counted using a Countess II automated cell counter (Thermo Fisher Scientific).

4.1.4. Effects of GTS-21 on Treg development in TCR-activated T cells

To investigate the effect of GTS-21 on Treg development, portions of the cells (3×10^4 cells) from 10 specimens were cultured for 5 days in triplicate in a 24-well plate containing 2 ml of the standard medium with 5 ng/ml TGF-β and Human T-activator CD3/CD28 Dynabeads at a beads-to-cell ratio of 1:1 with or without 30 µM GTS-21.

4.2. Real-time PCR

Total mRNA was extracted from human CD4⁺ T cells using Sepasol RNA II Super (Nacalai Tesque), after which cDNAs were prepared by reverse transcription using a Prime Script RT reagent Kit (Takara Bio.) in a S1000 Thermal Cycler (Bio-rad). Real-time PCR analysis was conducted using TB Green Premix Ex Taq II, and predesigned primers (Takara Bio) with a Thermal Cycler Dice Real Time System (Takara Bio.). The sequences and catalog numbers of the predesigned primers were as follows: for *CHRNA7* (HA164722), 5'-TGGCCAGATTTGGAAACCAGA-3' (sense) and 5'-AGTGTGGAATGTGGCGTCAAAG-3' (anti-sense); for *CHRFAM7A* (HA137753), 5'-GGTCAAGGCCAAACCGAAG-3' (sense) and 5'-TCCTGCTGACTCAGGTGTCCA-3' (anti-sense); and for GAPDH (HA067812), 5'-GCACCGTCAAGGCTGAGAAC-3' (sense) and 5'-TGGTGAAGACGCCAGTGGA-3' (anti-sense).

CHRNA7 and *CHRFAM7A* expression in each individual was normalized by GAPDH expression. Then to compare the magnitude of interindividual variability in *CHRNA7* and *CHRFAM7A* expression in resting CD4⁺ T cells, the levels of *CHRNA7* and *CHRFAM7A* expression in each individual were divided by the values closest to their respective medians and plotted in Figure 1.

To investigate the changes in *CHRNA7* and *CHRFAM7A* expression during TCR activation, values observed in each individual after various treatments and culture periods were compared with those observed in controls on day 1, after frozen/thawed samples had adapted to culture medium [67].

4.3. Flow cytometry for Treg development

To detect Tregs on day 5 of culture, cells were first washed with Hanks' balanced salt solution supplemented with 0.1% bovine serum albumin and 0.1% NaN₃, then stained using FITC-conjugated anti-CD4 antibody (RM4.5, Thermo Fisher Scientific) and PE-conjugated anti-CD25 antibody (PC61.5, Thermo Fisher Scientific). After subsequent fixation and permeabilization using BD Cytofix/Cytoperm solution (BD Biosciences), the cells were further stained with APC-conjugated anti-FoxP3 antibody (3G3, Thermo Fisher Scientific) and subjected to flow cytometric analysis (CytoFLEX, Beckman Coulter). A gate was set on the lymphocytes using characteristic forward scatter (FSC) and side scatter (SSC) parameters. Isotype-matched FITC-, PE- and APC-conjugated mouse IgG₁ Abs were used as controls. The acquired data was analyzed using CytExpert (Beckman Coulter).

4.4. Statistical analysis

Data are presented as means \pm S.E.M. Statistical analyses were performed using SPSS (IBM). Difference of the standard deviation was assessed using F-test. Differences between two groups were evaluated using paired t-test, and between three or more groups using two-way analysis of variance (ANOVA) with *post-hoc* Tukey's test, respectively. Values of $p < 0.05$ were considered as significant.

Author Contributions: KK: MM, TF and TK were involved in study design, interpretation of the results, writing and revising the manuscript; MM and TF performed experiments; SO, HM, YM, and TA were participated in revising the manuscript; KK, MM, TF, SO, YM, HM, and TA reviewed and approved the final version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from MM (mmashimo@dwc.doshisha.ac.jp) if and when such request is deemed appropriate and justified.

Conflicts of Interest: The authors declare no conflict of interest.

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