Dasatinib-induced anti-leukemia cellular immunity through a novel subset of CD57 positive helper/cytotoxic CD4 T cells in chronic myelogenous leukemia patients

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Abstract

Dasatinib has been known to induce lymphocytosis of large granular lymphocytes (LGLs) in a proportion of patients with chronic myelogenous leukemia (CML), and is associated with better clinical outcomes. LGLs consist of cytotoxic T lymphocytes and natural killer cells; however, the context and phenotypic/functional features of each type of LGL are unknown. To further define features of these LGLs, we investigated lymphocytosis in CML patients treated with dasatinib. D57-positive CD4-positive type I T helper (Th)-cells ($CD57^+$ Th-cells) rarely occur in CML patients without lymphocytosis and in healthy individuals; however, a substantial increase in the proportion of $CD57^+$ Th-cells was observed in CML patients treated with dasatinib. Additionally, these cells showed appreciable levels of cytocidal activity via cytotoxic degranulation. Analysis of T-cell receptor α and β sequences showed a skewed T-cell repertoire in the $CD57^+$ Th-cells. Furthermore, patients with LGLs and $CD57^+$ Th-lymphocytosis achieved stronger molecular responses than did those without lymphocytosis. Therefore, while further studies are warranted, our observations suggest that dasatinib induces the expansion of $CD57^+$ Th-LGLs, which may play a crucial role in the dasatinib-induced response against Philadelphia chromosome-positive leukemia.

Key words: CD57 expression, chronic myelogeneous leukemia, cytotoxic $CD4^+$ T-cell, dasatinib, large granular lymphocyte

Introduction

Chronic myelogenous leukemia (CML) is a hematopoietic stem cell disease characterized by a translocation between chromosome 9 and chromosome 22 (the Philadelphia chromosome [Ph]), leading to the formation of the oncogenic *BCR-ABL* fusion gene. The treatment for chronic phase (CP)-CML has changed dramatically since the clinical application of the first-generation tyrosine kinase inhibitor (TKI) imatinib. The current TKI-based treatment is efficacious, with favorable outcomes in most patients with CP-CML [1, 2]. Dasatinib is a widely used second-generation TKI, indicated for the treatment of newly diagnosed CML, CML with resistance or intolerance to prior treatment, and Ph-positive acute lymphoblastic leukemia [3]. Using second-generation TKIs, patients tended to achieve faster and better clinical responses without disease progression [4, 5]. Such favorable outcomes have prompted the prolonged use of TKIs, resulting in the emergence of various drug-induced off-target effects and adverse events (AEs). TKI-induced off-target effects have received much attention recently, as researchers have aimed to achieve greater clinical efficacy and circumvent AEs associated with TKIs [6].

An elevation in the number of peripheral blood (PB) large granular lymphocytes (LGLs) is one of the well-known off-target effects of dasatinib, and is observed in almost 30% of dasatinib-treated cases [7, 8]. This elevation in LGLs is thought to be mainly due to the expansion of LGLs which display cell-surface $CD3^+$ $CD8^+$ (markers for cytotoxic T lymphocytes, CTLs) or $CD16^+$ $CD56^+$ (markers for natural killer cells, NK cells) markers [8, 9]. In this context, these expanded LGLs may reasonably be assumed to have therapeutic activity against Ph-positive leukemic cells through their cytocidal potential [7, 8]. In practice, when compared to patients without LGL

lymphocytosis, those with LGL lymphocytosis tend to exhibit better clinical outcomes. This clinical observation that LGLs concurrently display anti-CML activity and precipitate damage to normal tissue strongly suggests an interposition of an aberrant immune response evoked by dasatinib [7, 8, 10]. These effects may be explained by evidence which shows that, in addition to *BCR-ABL*, dasatinib inhibits several key regulators of immune responses such as the Src tyrosine kinase [11-13]. However, the exact mechanisms of dasatinib-induced immune-modulatory effects have yet to be clarified.

Herein, we report a significant expansion of a novel and unique subset of LGLs, the CD57-positive CD4-positive type I T helper (Th)-cells ($CD57^+$ Th-cells), in a sub-population of CML patients undergoing dasatinib treatment. In this study, we aimed to define phenotypic and functional features of dasatinib-induced $CD57^+$ Th-cells in detail. We evaluated the impact of the LGL expansion on clinical outcomes, and discuss our findings in the context of relevant literature. Based on our observations, we hypothesize that $CD57^+$ Th-cells expand in CML patients treated with dasatinib and represent potential targets for cellular immunotherapy against CML.

Materials and methods

Acquisition and analysis of samples from CP-CML patients

Twenty-two patients diagnosed with CP-CML who were treated with dasatinib (dose range 70–100 mg/day) at the Juntendo University School of Medicine between September 2012 and August 2015, were analyzed. PB was drawn from all patients approximately 2 hours after dasatinib administration [14], when the drug plasma concentrations were at their peak levels. According to previously published studies,

LGL lymphocytosis has been diagnosed on the basis of an increased number of PB lymphocytes ($\geq 3.0 \times 10^9$/L) with morphological characteristics of LGLs in PB smear samples. An increased number of $CD57^+$ Th-cells was defined as an absolute count $\geq 0.3 \times 10^6$/L in this study. The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee at the Juntendo University School of Medicine (UMIN ID: UMIN000006501). Written informed consent for the use of samples and clinical records was obtained from all patients before sample collection.

Separation of mononuclear cells

PB was drawn from patients at diagnosis and at several time points (3, 6, 9, and 12 months) during dasatinib treatment. PB mononuclear cells (PBMCs) were separated from 20 mL of PB using the Lympho separation medium (MP Biomedicals, Solon, Ohio, USA), and stored at -80°C or in liquid nitrogen until use.

Immunophenotypic analyses

Flow cytometric analysis of PBMCs was performed using the following antibodies: APC-anti-CD3 (SK7, BD Biosciences, Franklin Lakes, New Jersey, USA), APC-H7-anti-CD4 (RPA-T4, BD Biosciences), PE-CF594-anti-CD8 (RPA-T8, BD Biosciences), PE-Cy7-anti-CD16 (3G8, BD Biosciences), PE-anti-CD56 (MY31, BD Biosciences), FITC-anti-CD57 (HNK-1, BD Biosciences), PE-anti-interleukin (IL)-4 (MP4-25D2, BD Biosciences), PE-Cy7-anti-IL-17A (eBio64DEC17, eBioscience, San Diego, California, USA), Alexa Flour 700-anti-IFN-γ (B27, BD Biosciences), PE-anti-TCR Vα24-Jα18 (6B11, BioLegend, San Diego, California, USA), PE-Cy7-anti-KIR (DX27, BioLegend), PE-Vio770-anti-NKG2A (REA110, Miltenyi

Biotec, Bergisch Gladbach, Germany), PerCP-Cy5.5-anti-NKG2D (1D11, eBioscience), and PE-Vio770-anti-DNAM-1 (DX11, Miltenyi Biotec). Patient-derived PBMCs were sorted into six subpopulations for use as effector cells. The $CD3^-$ $CD16^+$ $CD56^+/57^+$ populations were defined as NK cells, and the $CD3^+$ $CD4^+$ $CD57^{+/-}$ and $CD3^+$ $CD8^+$ $CD57^{+/-}$ populations as T-cells. Cell sorting was performed using a FACS Aria II cell sorter (BD Biosciences). Flow cytometry was performed using a BD LSR Fortessa (BD Biosciences). Data were analyzed with the FlowJo software (Tree Star).

Intracellular cytokine staining

For intracellular cytokine staining, the sorted effector cells were cultured with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) and ionomycin (ION) (500 ng/mL) (Sigma-Aldrich, St. Louis, Missouri, USA) for 12 hours, with the addition of Brefeldin A (Sigma-Aldrich) for the last 2 hours of incubation. The cells were then fixed with 4% paraformaldehyde, permeabilized with permeabilization buffer (eBioscience, San Diego, California, USA), and stained with antibodies.

Chromium-51 release assay of LGLs against K562 cells

Cytocidal activity mediated by patient-derived LGLs against K562 cells lacking HLA molecules (K562: a *BCR-ABL*-positive leukemia cell line) was determined using the standard 4-hour chromium-51 release assay (^{51}Cr-release assay). Cytocidal activity was assessed in six sorted subpopulations of LGLs (used as effector cells), and a comparative assessment was made. Results were calculated and recorded as percentage of dead cells, as described previously [15]. In certain experiments, K562 cells transduced with HLA-A24 (K562-A24), or those expressing both HLA-24 and CIITA

gene-modified HLA class II molecules (K562-A24/C II TA), were similarly employed as target cells [16].

Gene expression analysis

The expression levels of the granzyme B and perforin genes in the sorted effector cells were quantified by quantitative polymerase chain reaction (qPCR). Total RNA was extracted from the sorted cells using the QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was analyzed by qPCR performed using the THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) in a CFX96 real-time system (BioRad, Hercules, California, USA). The following primers were used: granzyme B, forward: TGCAGGAAGATCGAAAGTGCG, reverse: GAGGCATGCCATTGTTTCGTC;, perforin, forward: CGCCTACCTCAGGCTTATCTC, reverse: CCTCGACAGTCAGGCAGTC; and glyceraldehyde 3-phosphate dehydrogenase. forward: AGAGACCCTCACTGCTG, reverse: AGATTCAGTGTGGTGGG. Quantification was performed by using the relative standard curve method against the control gene (glyceraldehyde 3-phosphate dehydrogenase).

Diversity of T-cell receptor (TCR) α and TCRβ analyzed by deep sequencing

Total RNA was extracted from $CD57^{+/-}$ Th-cells in CML patients who developed LGL lymphocytosis, and quantified (QIAamp RNA Blood Mini Kit, Qiagen, Venlo, Netherlands). T-cell receptor (TCR)α and TCRβ chain sequencing was performed at Repertoire Genesis Incorporated (Osaka, Japan) by using the unbiased gene amplification method with adaptor-ligation PCR. An average of 2.1×10^5 unique reads

were generated for the $CD57^{+/-}$ Th-cell samples. Bioinformatics analysis was performed on the sequencing data by using a repertoire analysis software (Repertoire Genesis, Repertoire Genesis Incorporated, Osaka, Japan). A program for sequence homology searches was implemented using BLASTN, an automatic aggregation program, a graphics program for use of TRV and TRJ, and CDR3 length distribution. Sequence identities at the nucleotide level between query and entry sequences were automatically calculated.

Statistical analysis

All P-values of 0.05 or less were considered statistically significant. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [17], a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). EZR is a modified version of the R commander, designed to add statistical functions frequently used in biostatistics.

Results

Occurrence of LGL lymphocytosis and treatment-associated AEs

Among the 22 patients studied, 11 (50%) developed LGL lymphocytosis as confirmed using light microscopy. During dasatinib treatment, the median time to lymphocytosis was 6 months from the start of treatment (range, 3–24 months). The peak lymphocyte count ranged from 3.0×10^9/L to 9.6×10^9/L. In most patients, LGL lymphocytosis was long-lasting and continued throughout dasatinib treatment, albeit with fluctuations in the absolute lymphocyte number. In the 11 patients who developed LGL lymphocytosis, 5 had pleural effusion, and 1 developed skin rash. Among these 11

patients, 1 patient showed cytomegalovirus (CMV) reactivation with positivity for both CMV-IgM and CMV antigen C10/C11 (13/20 cells). The incidence of each AE was similar in groups with or without lymphocytosis (Table 1).

Phenotypic and functional features of LGLs

Induction and phenotypic features of CD57$^+$ effector memory Th-cells by dasatinib

Flow cytometric analysis of PBMCs revealed that compared to those without lymphocytosis, patients with LGLs showed a substantial increase in CD56$^+$ NK cells, CD57$^+$ NK cells, and CD57$^+$ CTLs (similar to previous reports [8]) (Fig 1A-C). Furthermore, CD57-expressing cells contained an increased proportion of CD4$^+$ T-cells (Fig 1D). Immuno-phenotypes of LGLs in a representative case are shown in Fig S1A-H. Unlike CD57$^-$ Th-cells, almost all CD57$^+$ Th-cells were positive for cytoplasmic granules (1/100 cells vs. 99/100 cells, respectively) (Fig S2A-B). These unique CD57$^+$ Th-cells displayed a CD45RA$^-$, CCR7$^-$ effector memory T-cell phenotype (Fig S3A), were positive for NK cell adhesion molecule DNAM-1, and were negative for killer immunoglobulin-like receptor (KIR) and NKG2D. The mean fluorescence intensity (MFI) of DNAM-1 on CD57$^+$ Th-cells tended to be higher than that on CD57$^-$ Th-cells (592.7 $\pm$ 97.6 vs. 392 $\pm$ 180.2, respectively, n=3) (statistically insignificant, probably due to small number of cases analyzed (data not shown)). Furthermore, CD57$^+$ Th-cells did not display the TCR Vα24-Jα18 chain, and thus were not invariant NK/T cells (Fig S3B). In addition, neither CD3$^+$CD4$^-$CD57$^+$ cells nor CD57$^-$ Th-cells displayed TCR Vα24-Jα18 (Fig S3C-D) [18]. In contrast to unstimulated CD57$^+$ Th-cells (Fig S4A-B), CD57$^+$ Th-cells stimulated with PMA/ION produced IFN-γ, but not IL-4 or IL-17A (Fig S4C-D). This observation suggested that

$CD57^+$ Th-cells were potentially type 1 helper $CD4^+$ T cells. A substantial expansion of unique $CD57^+$ Th-cells with LGL morphology was observed in all CML patients with LGL lymphocytosis.

$CD57^+$ Th-cells exert cytocidal activity against NK cell-sensitive target cells

Because CD57 is displayed by circulatory immune cells with natural killer activity, we next examined the cytocidal activity mediated by $CD57^+$ Th-cells against K562, a popular NK cell-sensitive *BCR-ABL*-positive human cell line (3 different variants were used: K562, K562-A24/CIITA, and K562-A24), using a standard ^{51}Cr-release assay. $CD3^-$ $CD16^+$ $CD56^+$ NK cells (Fig 2A), $CD3^-$ $CD16^+$ $CD57^+$ NK cells (Fig 2B) and $CD57^+$ CTLs (Fig 2C) displayed substantial cytocidal activity against K562 cells, as expected. $CD57^+$ Th-cells also exerted a certain level of cytotoxic activity against all variants of K562 cells in a cell number-dependent manner ((Fig 2D, S5), whereas neither $CD57^-$ Th-cells nor $CD57^-$ CTLs displayed cytocidal activity against any K562 variant (Fig 2C-D, S5). In addition, RT-PCR data showed a higher expression of both granzyme B (Fig 3A) and perforin (Fig 3B) (responsible for cytocidal activity) in $CD57^+$ Th-cells, compared to that in $CD57^-$ Th-cells; the expression of these molecules was similar in $CD57^+$ and $CD57^-$ NK cells and CTLs.

Oligoclonal expansion of $CD57^+$ Th-cells

We examined the diversity of TCRα and TCRβ sequences in $CD57^-$ and $CD57^+$ Th-cells in the PBMCs of three CML patients with lymphocytosis using deep sequencing analysis. Fig 4A-F shows the percentage of the top 20 TCR sequence reads in the respective cell populations. As shown, contrary to $CD57^-$ Th-cells, $CD57^+$

Th-cells demonstrated a highly skewed TCRα and TCRβ gene expression pattern. Surprisingly, the most common TCRα sequence in patient 3 was found in 42% of the $CD57^+$ Th-cells (Fig 4C). Some of the TCRα and TCRβ sequences were shared by both the $CD57^-$ and the $CD57^+$ Th-cell populations in individual patients. These results suggested the oligoclonal expansion of a limited number of T-cell clones selectively in the $CD57^+$ Th-cell population.

Correlation between LGL lymphocytosis and response to dasatinib treatment in CML patients

We examined the association of LGL lymphocytosis with dasatinib treatment response in CML patients. Nine of 11 (82%) patients with LGL lymphocytosis achieved a better molecular response characterized by a 4.0-log reduction from a standardized baseline ($MR^{4.0}$ *BCR-ABL* on the international scale ≤ 0.01%) within 24 months, whereas only 4 of 11 (36%) patients without LGL lymphocytosis achieved $MR^{4.0}$ (Fig 5A). Moreover, the expansion of $CD57^+$ Th-cells also showed a similar impact on prognosis. Six of 7 (86%) patients with $CD57^+$ Th-cell expansion achieved $MR^{4.0}$ within 24 months, whereas 10 of 15 (67%) patients without $CD57^+$ Th-cells expansion achieved $MR^{4.0}$ within 24 months (Fig 5B). While LGL lymphocytosis was associated with a better clinical response, the above data suggests that the dasatinib-induced expansion of $CD57^+$ Th-cells was also associated with possible anti-CML activity.

Discussion

In this report, we described the emergence of novel $CD57^+$ helper/cytotoxic $CD4^+$ T-cells ($CD57^+$ Th-cells) with typical LGL morphology in CML patients treated with

dasatinib. This T-cell subset was extremely rare in both healthy volunteers and in dasatinib-treated CML patients without LGL lymphocytosis.

CD57 is a surface molecule expressed by a proportion of T-cells and NK cells [19, 20]. In previous reports, the expansion of $CD4^+$ $CD57^+$ T-cells was associated with tuberculosis, malaria, rheumatoid arthritis, and HIV-1 infection [21-25]; however, the cell populations expanded in the above mentioned diseases have been categorized as terminally differentiated effector T cells, distinct from the effector memory T-cell populations observed in dasatinib-treated patients. To date, the biological significance of CD57 is unclear, as is the function of $CD57^+$ Th-cells. Hence, this study is the first attempt to define the function of this unique immune cell population which is expanded during dasatinib treatment.

In our study, the expanded $CD57^+$ Th-cells consisted of α/β $CD4^+$ T-cells, while invariant NK/T cells bearing TCR-Vα24/Jα18 expression were absent. The expanded cells produced type I proinflammatory cytokines when stimulated with PMA/ION, and exerted moderate but significant NK-cell like cytocidal activity against NK-sensitive *BCR-ABL*-positive K562 cells in a cell number-dependent manner. $CD57^+$ Th-cells also killed K562-A24 and K562-A24/C II TA, and showed higher expression of granzyme B and perforin. These results indicated that the cytocidal activity mediated by $CD57^+$ Th-cells *in vitro* was irrelevant to the recognition of the target epitope/HLA class II complex via TCR, although we did not explore this further with blocking experiments.

The $CD57^+$ Th-cells expressed DNAM-1, an adhesion molecule reported to mediate cytocidal activity in NK cells [26]. Interestingly, K562 cells are known to express substantial amounts of CD155 (Polio Virus Receptor), the ligand for DNAM-1 [27]. In this study, dasatinib-treated patients with expanded $CD57^+$ Th-cells displayed a

statistically better clinical response compared to those without such an expansion. Further studies are needed to clarify whether NK-cell like cytocidal activity involving the DNAM-1/CD155 pathway plays a role in the anti-CML effect of dasatinib.

Interestingly, compared to $CD57^-$ Th-cells, $CD57^+$ Th-cells displayed a $CD45RA^-/CCR7^-$ effector memory T-cell phenotype and a highly skewed TCRα/β repertoire. These findings imply that the expanded $CD57^+$ Th-cells are likely to recognize distinctive antigens. Since our results show that $CD57^+$ Th-cells may have a positive prognostic impact, it is possible that a subset of $CD4^+$ helper T-cells capable of recognizing undetermined CML-related antigens may be induced to express CD57, and these cells contribute to anti-CML effects during dasatinib treatment. Ueda et al. reported that *BCR-ABL*-specific $CD4^+$ helper T-cells induced CML-reactive CTLs with the support of dendritic cells *in vitro* [29]. This report supports our hypothesis, even though we could not define the target antigens of the $CD57^+$ Th-cells in this study.

Dasatinib acts mainly by blocking *BCR-ABL* kinase activity; however, several off-target effects have been identified. For instance, dasatinib inhibits Src-family kinases such as lymphocyte-specific tyrosine kinase (Lck) and Fyn. At clinically relevant doses, inhibition of Src-family kinases has been reported to impair T-cell function [30]. These findings account for the immuno-suppressive complications (including CMV reactivation) associated with dasatinib therapy [28-31]. However, in this study, serological examination revealed that the induction of $CD57^+$ Th-cells by dasatinib was not associated with CMV-IgG status. Additionally, CMV-reactive $CD57^+$ $CD4^+$ T cells have been described to execute KIR-mediated cytocidal activity [32]. Taken together, the above evidence suggests that expanded $CD57^+$ Th-cells in dasatinib-treated patients may be distinct from CMV-related T-cell populations. In

addition, the other known off-target effect of dasatinib at clinical doses is the suppression of $CD4^+CD25^+$ regulatory T-cells (T-regs) [33]. These dasatinib-induced, immune-related, off-target effects may potentially be involved in the expansion of $CD57^+$ Th-cells observed in our study; however, the implications of our findings should be addressed in further studies.

In conclusion, phenotypic and functional analyses of dasatinib-induced LGLs revealed the existence of a unique $CD57^+$ Th-cell population. $CD57^+$ Th-cell expansion may mediate a therapeutic activity against CML cells through cytokine secretion and cytotoxic activity, though the target antigens recognized by these cells are unknown. However, if the antigens are dasatinib-induced and/or leukemia cell-related, our findings would facilitate the development of a novel treatment strategy for CML patients, using a combination of dasatinib and CML-specific $CD4^+$ T cell-based cellular immunotherapy.

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Author Contributions

N.W. performed the study, analyzed the results, and wrote the paper; T. Takaku designed the research, analyzed the results, wrote the paper, and directed the research; K.T. and S.S. analyzed the results; T. Toyota performed the study; M.K., M.N., and T.H. contributed analytical materials; H.F. discussed and analyzed data, provided experimental concepts and materials, and edited the paper; and N.K. directed the

research.

Disclosure of Conflicts of Interest

The authors declare that they have no conflict of interest. A summary of relevant information will be published with the manuscript.

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Legends

Table I

Characteristics of the study patients.

Figure 1

Lymphocyte subpopulations in patients with and without large granular lymphocyte lymphocytosis.

Flow cytometric comparison of four subpopulations of lymphocytes among patients with and without large granular lymphocyte (LGL) lymphocytosis (n = 3 each). (A) $CD56^+$ natural killer (NK) cells. (B) $CD57^+$ NK cells. (C) $CD57^+$ cytotoxic T lymphocytes. (D) $CD57^+$ helper T-cells. Horizontal bars represent the median lymphocyte count in each subpopulation.

Figure 2

Large granular lymphocytes (LGLs) isolated from chronic myeloid leukemia patients who developed LGL lymphocytosis.

Analysis of cytotoxicity against K562 cells by chromium-51 release assay in the respective purified cell populations. (A) Cytotoxic activity of $CD56^+$ natural killer (NK) cells. (B) Cytotoxic activity of $CD57^+$ NK cells. (C) Comparison of the cytotoxicity of CD57 positive and negative CTLs. The solid line represents the cytotoxicity of $CD57^+$ CTLs and the broken line represents the cytotoxicity of $CD57^-$ CTLs. (D) Comparison of the cytotoxicity of CD57 positive and negative Th-cells. The solid line represents the cytotoxicity of $CD57^+$ Th-cells and the broken line represents the cytotoxicity of $CD57^-$ Th-cells. Error bars represent the standard errors of the means.

E/T, effector cells/target cells.

Figure 3

Gene expression of granzyme B and perforin of LGLs.

Gene expression of (A) granzyme B and (B) perforin in natural killer cells, cytotoxic T lymphocytes, and in helper T cells, as evaluated by quantitative polymerase chain reaction (qPCR).

Figure 4

T-cell receptor (TCR) α and TCRβ diversity analysis through deep sequencing.

Percentage of the top 20 TCR sequence reads from CD57-positive and -negative helper T-cells in three chronic myelogenous leukemia patients treated with dasatinib. TCRα gene ranking in (A) patient 1, (B) patient 2, and (C) patient 3. TCRβ gene ranking in (D) patient 1, (E) patient 2, and (F) patient 3.

Figure 5

Cumulative incidence of achieving molecular response after dasatinib treatment in chronic myelogenous leukemia patients.

Comparison of treatment response in patients (A) with and without lymphocytosis, and in patients (B) with and without expansion of $CD57^+$ helper T-cells.

Supplemental Figure 1

Representative immunophenotyping analysis of large granular lymphocytes (LGLs).

$CD56^+$ natural killer (NK) cells in patients (**A**) without and (**B**) with LGL lymphocytosis. $CD57^+$ NK cells in patients (**C**) without and with (**D**) LGL lymphocytosis. $CD57^+$ cytotoxic T lymphocytes in patients (**E**) without and (**F**) with LGL lymphocytosis. $CD57^+$ helper T-cells in patients (**G**) without and (**H**) with LGL

lymphocytosis.

Supplemental Figure 2

Comparison of cytoplasmic granules in CD57-positive and -negative Th-cells.

Cytoplasmic granules by Giemsa staining indicated morphologic features of sorted CD57 positive and negative helper T (Th)-cells. (**A**) $CD57^+$ Th-cells showed cytoplasmic granules. (**B**) CD57 negative Th-cells did not show cytoplasmic granules.

Supplemental Figure 3

Immunophenotyping analysis of $CD57^+$ Th-cells.

Flow cytometric analysis of representative T-cells. (**A**) Expression of CD45RA and CCR7. (**B**) interferon (IFN)-γ and interleukin (IL)-4. (**C**) IFN-γ and IL-17 in $CD57^+$ Th-cells. T-cell receptor (TCR) Vα24-Jα18 expression in (**D**) $CD57^+$ Th-cells, (**E**) $CD3^+$ $CD4^-$ $CD57^+$ cells, and in (**F**) $CD3^+$ $CD4^-$ $CD57^-$ cells.

Supplemental Figure 4

Analysis of intracellular cytokine production without stimulation in $CD57^+$ helper T (Th)-cells.

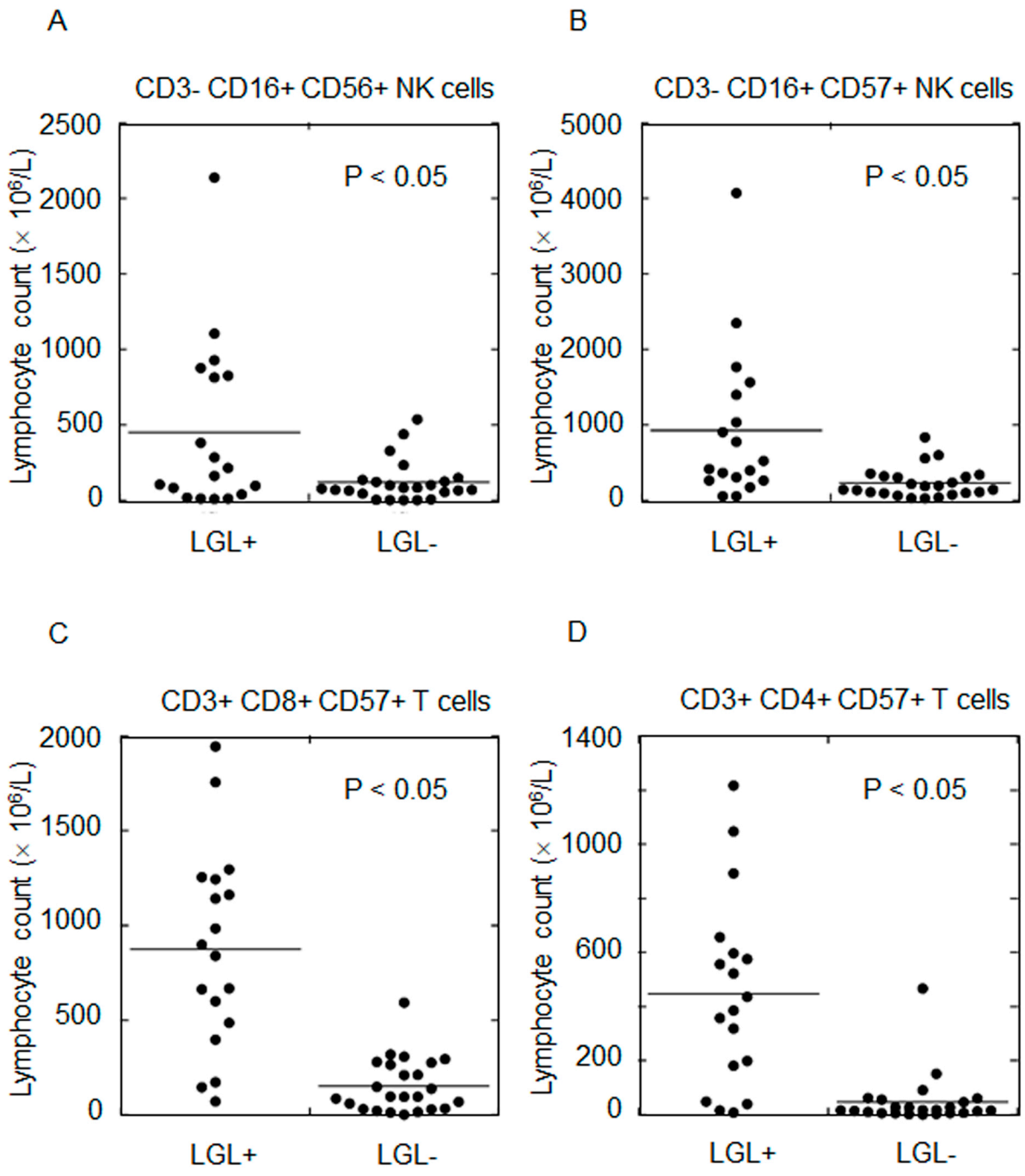
$CD57^+$ Th-cells without phorbol 12-myristate 13-acetate (PMA) and ionomycin (ION) stimulation did not produce any cytokine. (**A**) interferon (IFN)-γ and interleukin (IL)-4. (**B**) IFN-γ and IL-17. For intracellular cytokine staining, the sorted effector cells were cultured with PMA (50 ng/mL) and ION (500 ng/mL) (Sigma-Aldrich) for 12 hours, with the addition of Brefeldin A (Sigma-Aldrich) 2 hours before the end of incubation. The cells were then fixed with 4% paraformaldehyde, permeabilized with

permeabilization buffer (eBioscience), and stained with antibodies.

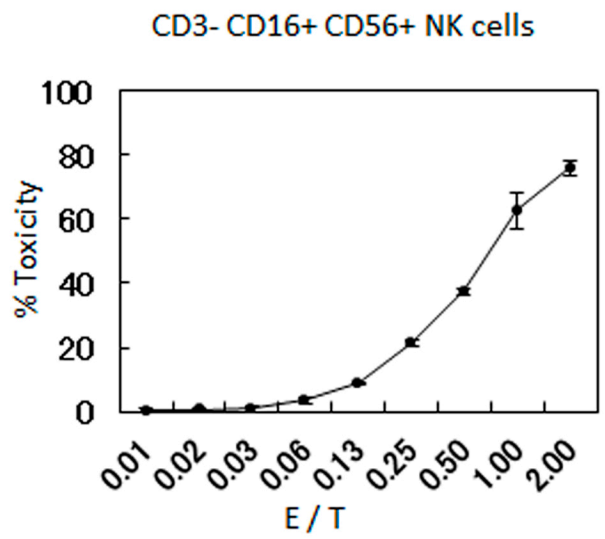
Supplemental Figure 5

Cytotoxicity of $CD57^{+/-}$ helper T (Th)-cells targeting K562-A24/C II TA and K562-A24.

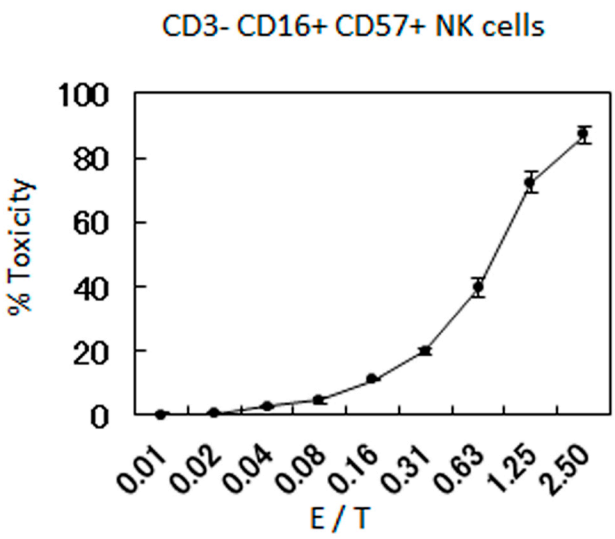
The solid line represents the cytotoxicity of $CD3^{+}$ $CD4^{+}$ $CD57^{+}$ Th-cells and the broken line represents the cytotoxicity of $CD3^{+}$ $CD4^{+}$ $CD57^{-}$ Th-cells.


A
CD3- CD16+ CD56+ NK cells
Lymphocyte count (× 10⁶/L)
2500
2000
1500
1000
500
0
P < 0.05
LGL+
LGL-
B
CD3- CD16+ CD57+ NK cells
Lymphocyte count (× 10⁶/L)
5000
4000
3000
2000
1000
0
P < 0.05
LGL+
LGL-
C
CD3+ CD8+ CD57+ T cells
Lymphocyte count (× 10⁶/L)
2000
1500
1000
500
0
P < 0.05
LGL+
LGL-
D
CD3+ CD4+ CD57+ T cells
Lymphocyte count (× 10⁶/L)
1400
1000
600
200
0
P < 0.05
LGL+
LGL-

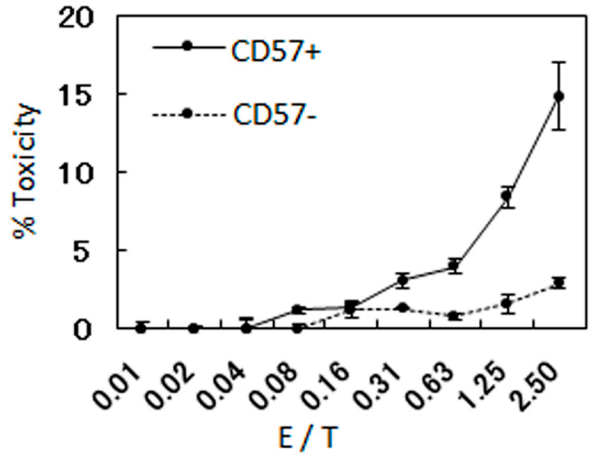
A


CD3- CD16+ CD56+ NK cells
% Toxicity
100
80
60
40
20
0
0.01
0.02
0.03
0.06
0.13
0.25
0.50
1.00
2.00
E / T

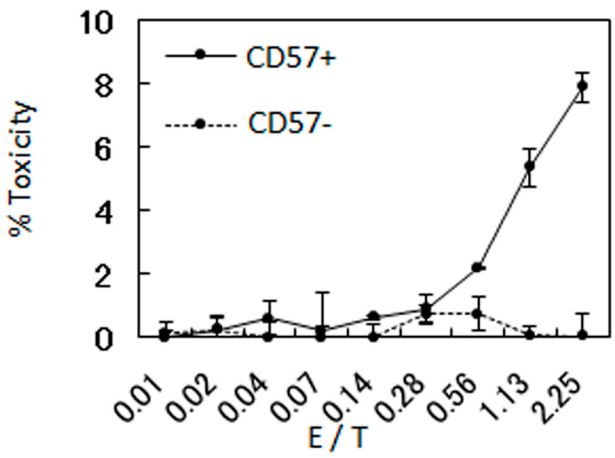
B


CD3- CD16+ CD57+ NK cells
% Toxicity
100
80
60
40
20
0
0.01
0.02
0.04
0.08
0.16
0.31
0.63
1.25
2.50
E / T

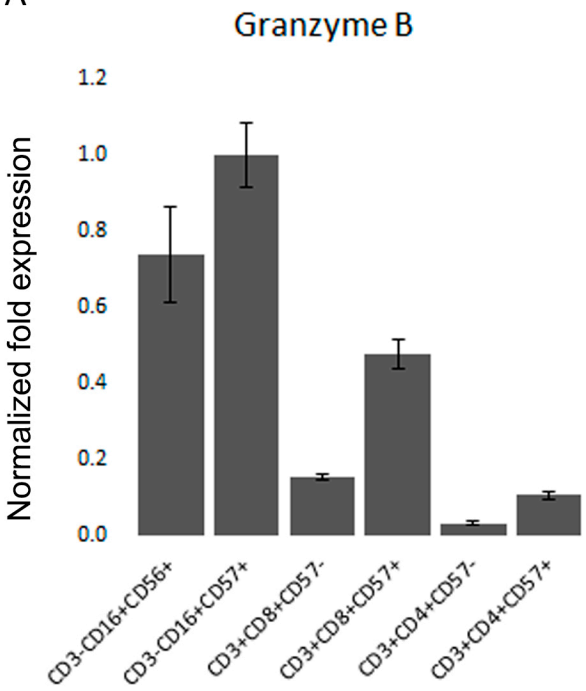
C


CD3+ CD8+ CD57+/- T cells
% Toxicity
20
15
10
5
0
CD57+
CD57-
0.01
0.02
0.04
0.08
0.16
0.31
0.63
1.25
2.50
E / T

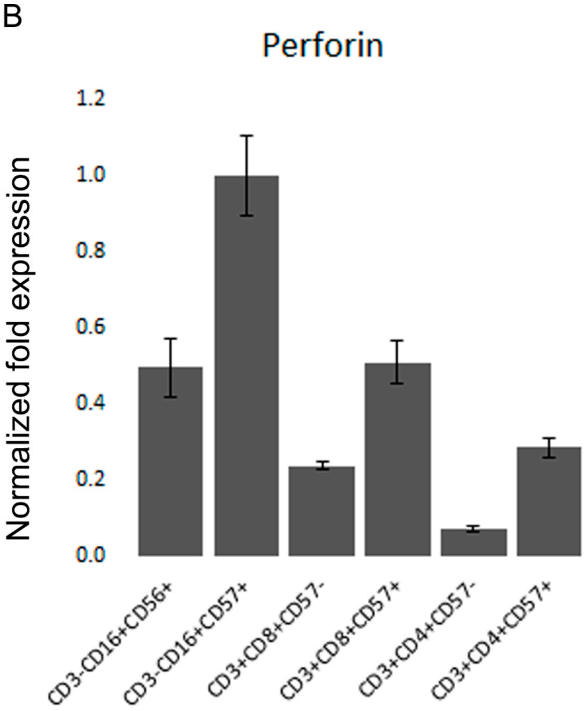
D

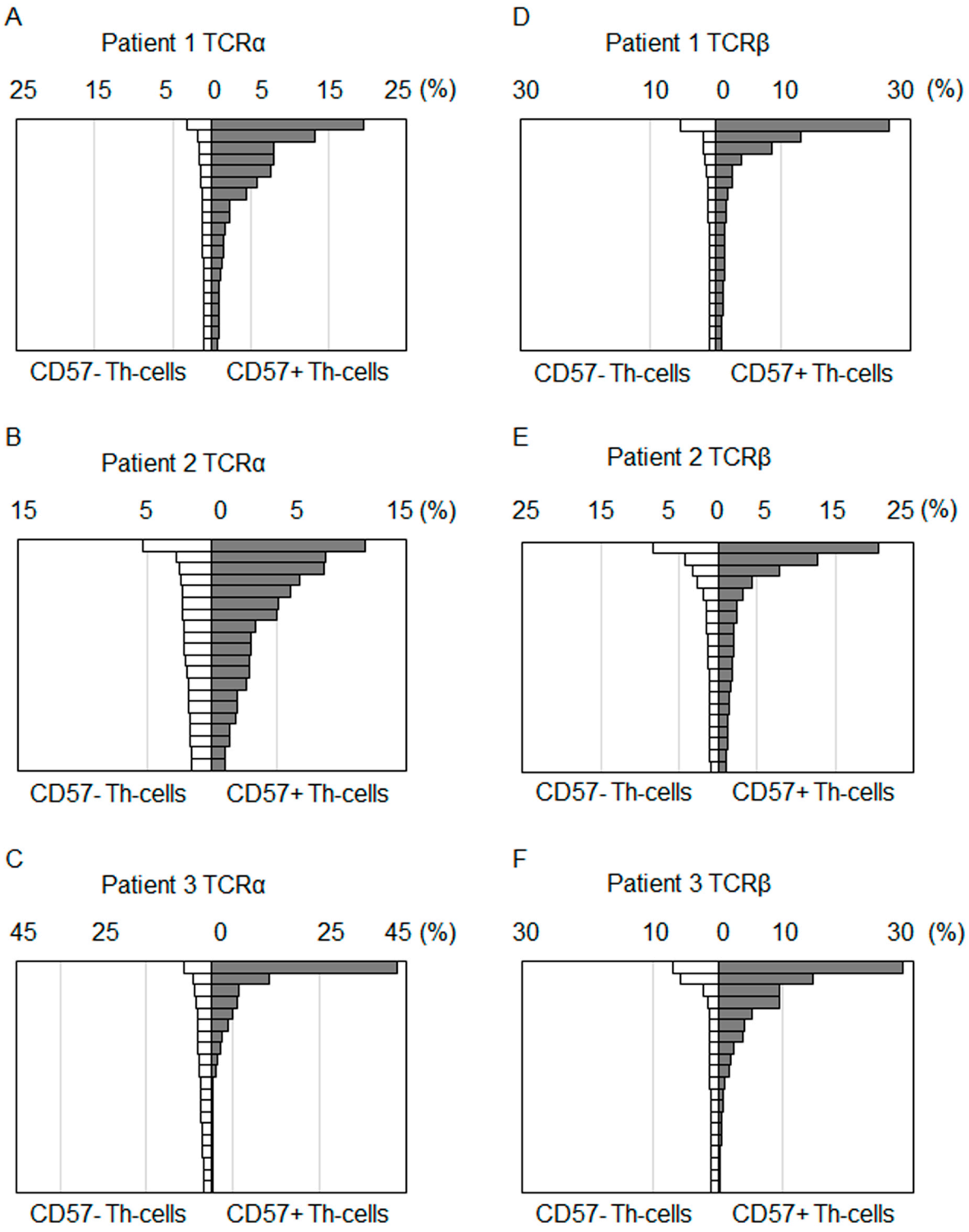

CD3+ CD4+ CD57+/- T cells
% Toxicity
10
8
6
4
2
0
CD57+
CD57-
0.01
0.02
0.04
0.07
0.14
0.28
0.56
1.13
2.25
E / T

A


Granzyme B
Normalized fold expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CD3-CD16+CD56+
CD3-CD16+CD57+
CD3+CD8+CD57-
CD3+CD8+CD57+
CD3+CD4+CD57-
CD3+CD4+CD57+

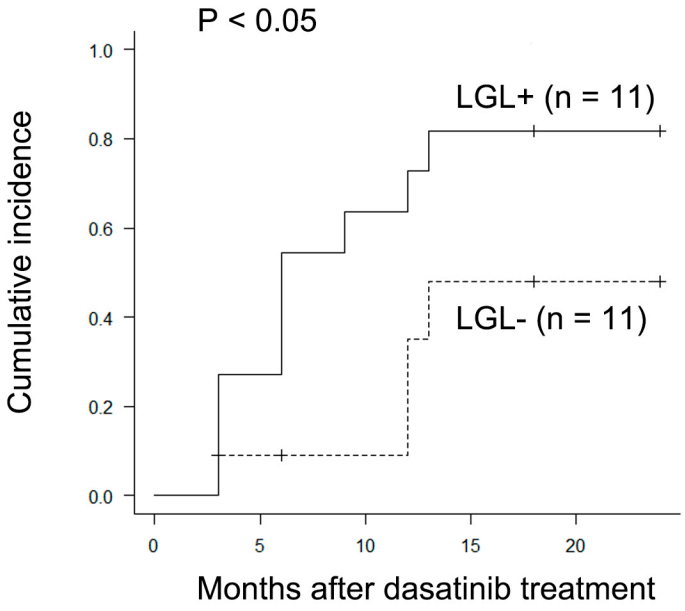
B


Perforin
Normalized fold expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CD3-CD16+CD56+
CD3-CD16+CD57+
CD3+CD8+CD57-
CD3+CD8+CD57+
CD3+CD4+CD57-
CD3+CD4+CD57+

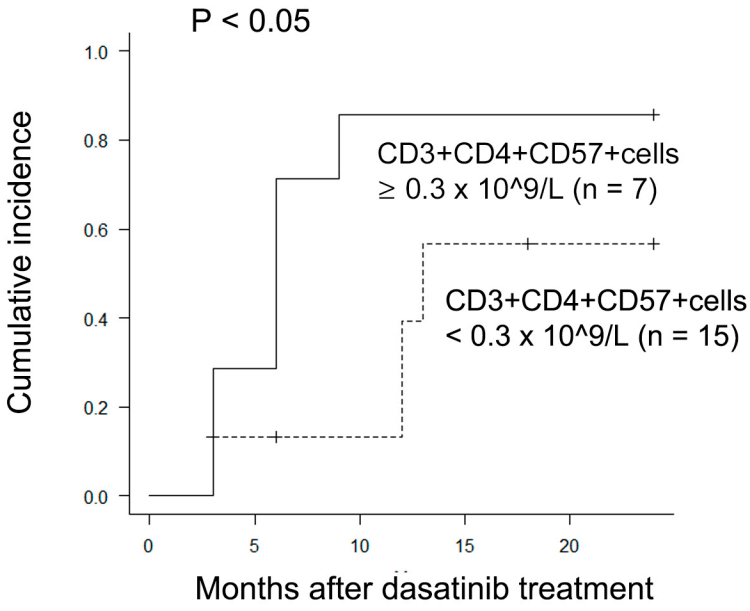

A
Patient 1 TCRα
25 15 5 0 5 15 25 (%)
CD57- Th-cells
CD57+ Th-cells
D
Patient 1 TCRβ
30 10 0 10 30 (%)
CD57- Th-cells
CD57+ Th-cells
B
Patient 2 TCRα
15 5 0 5 15 (%)
CD57- Th-cells
CD57+ Th-cells
E
Patient 2 TCRβ
25 15 5 0 5 15 25 (%)
CD57- Th-cells
CD57+ Th-cells
C
Patient 3 TCRα
45 25 0 25 45 (%)
CD57- Th-cells
CD57+ Th-cells
F
Patient 3 TCRβ
30 10 0 10 30 (%)
CD57- Th-cells
CD57+ Th-cells

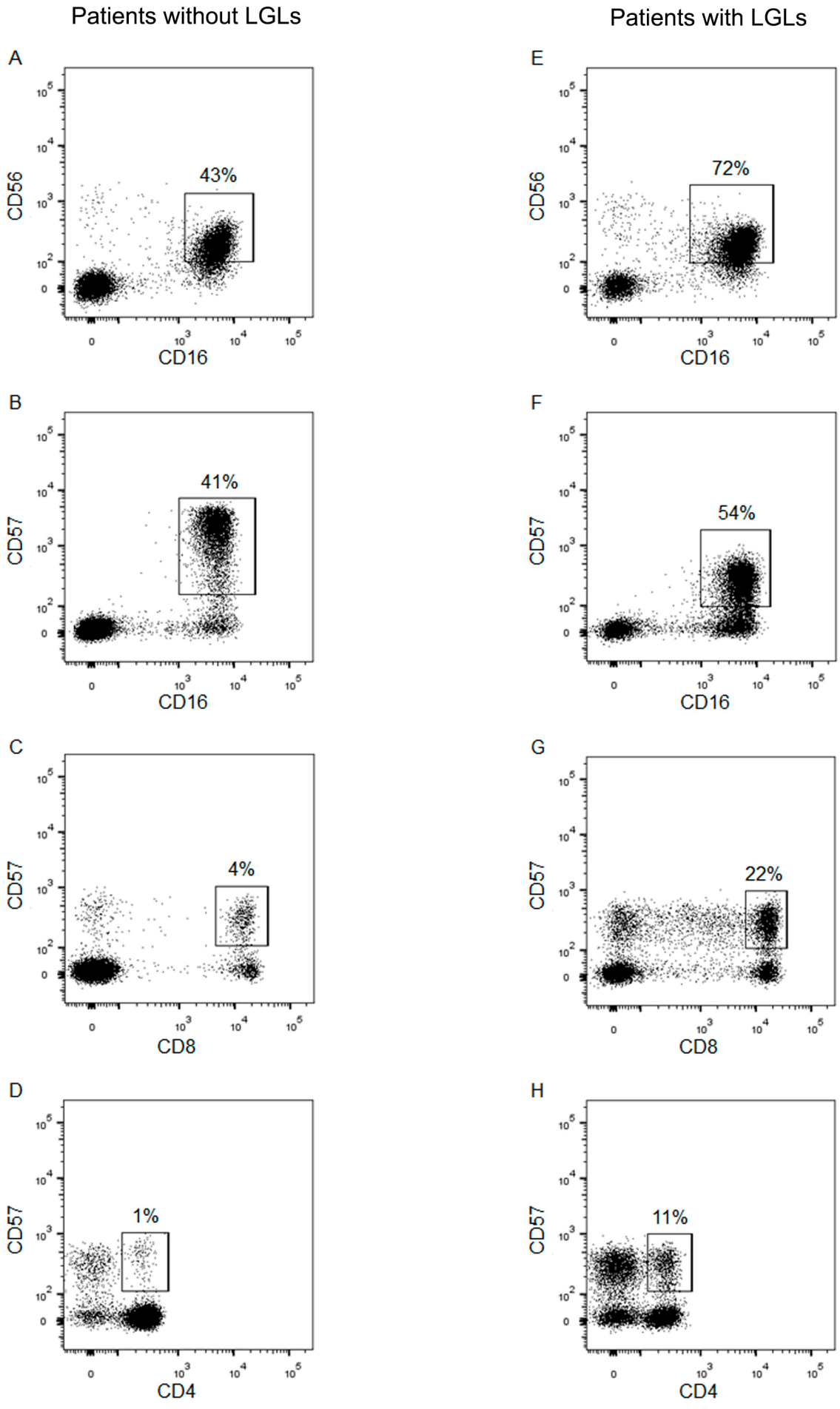
Watanabe et al., Figure 5

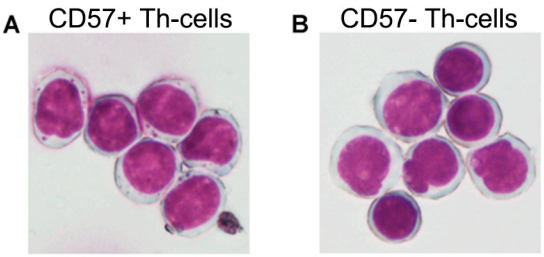
A

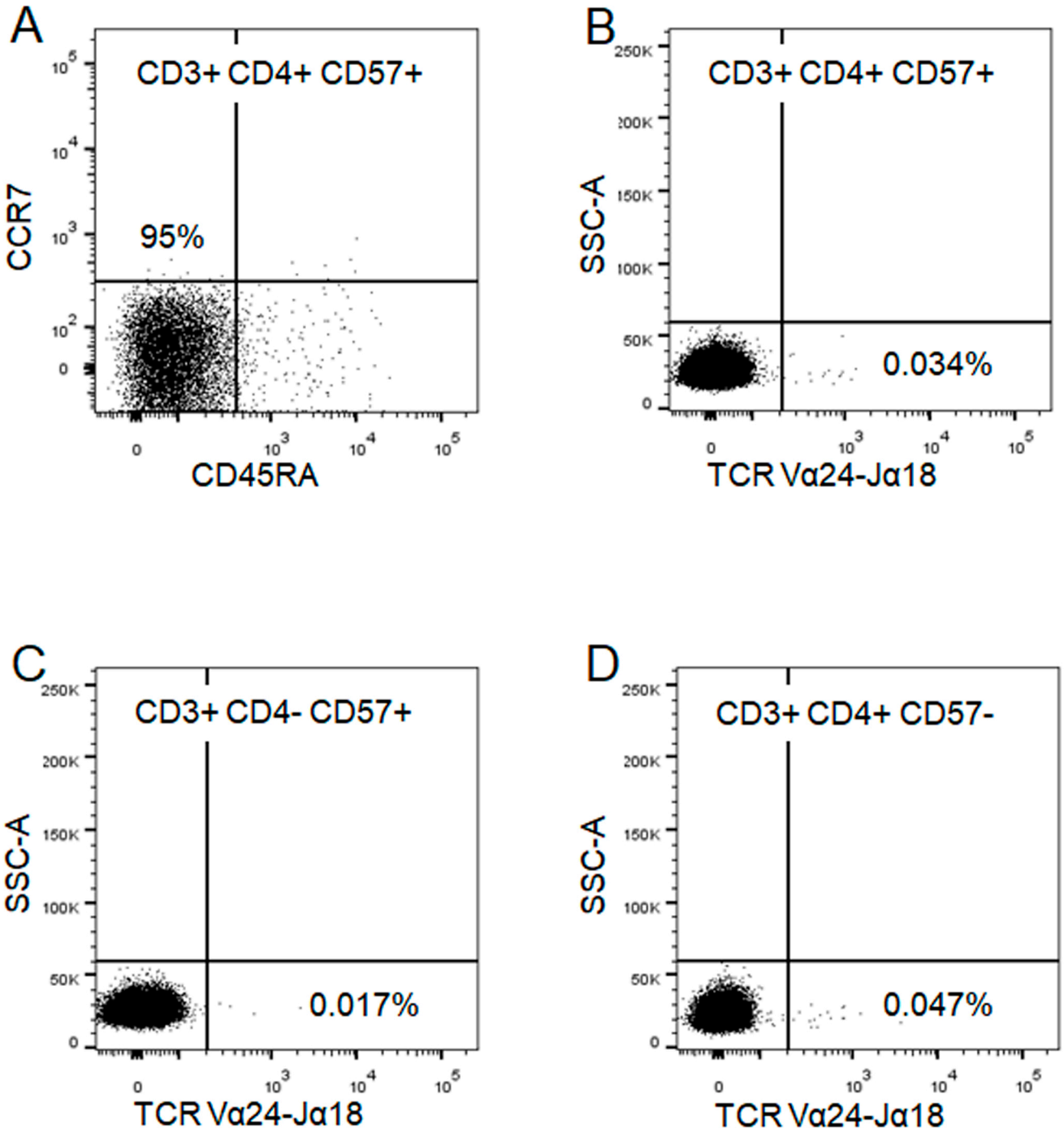


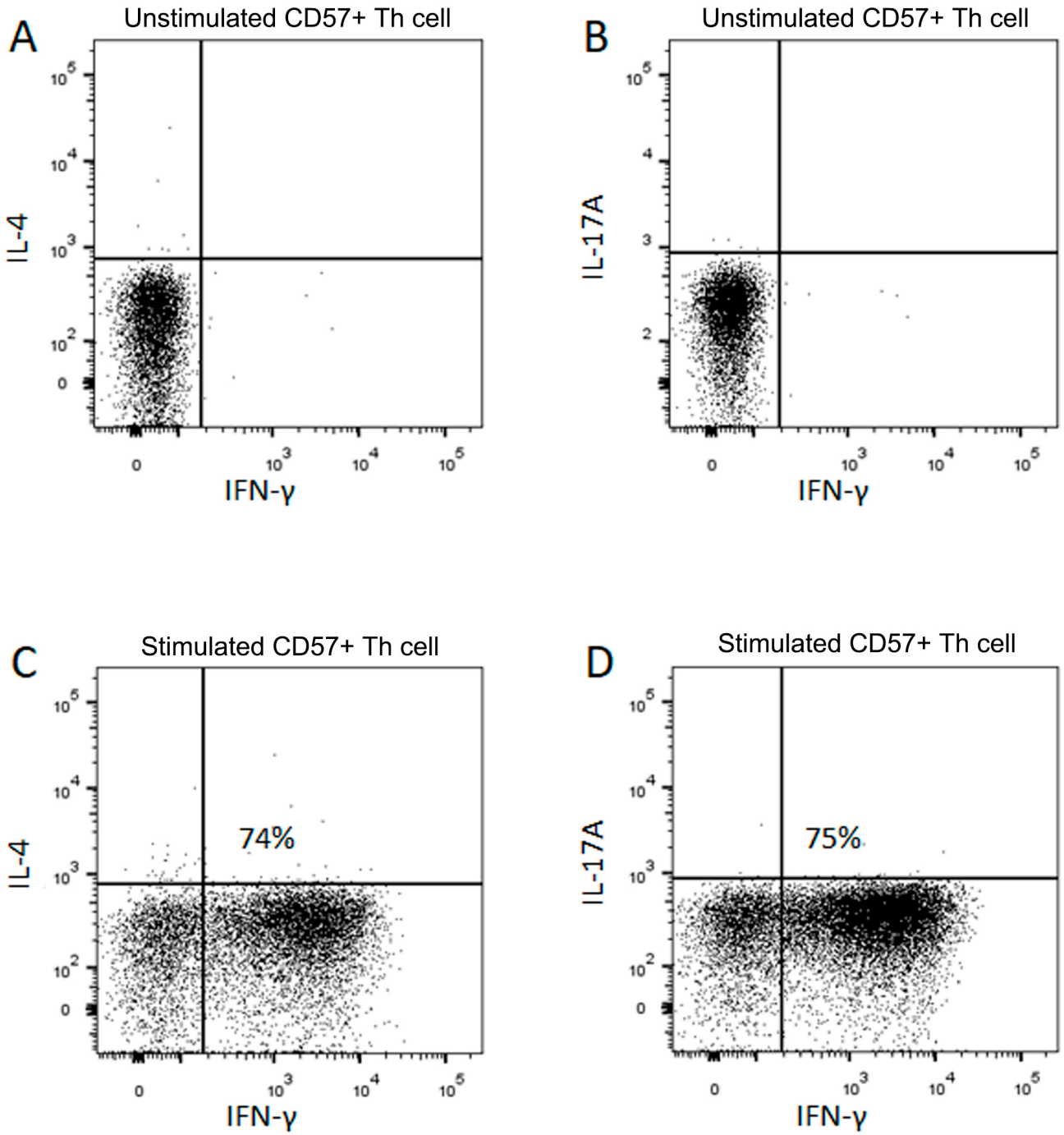
B

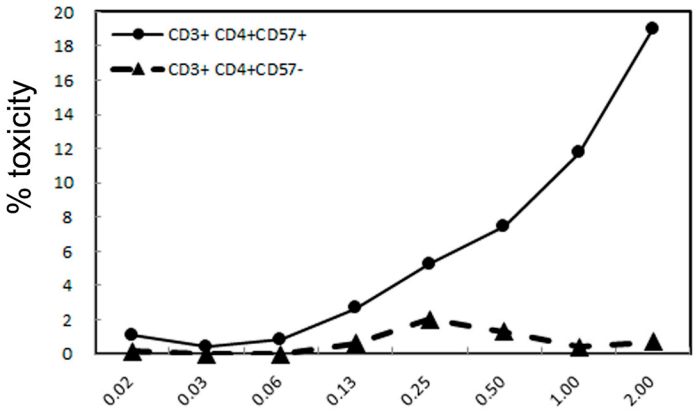



Patients without LGLs
Patients with LGLs
A
B
C
D
E
F
G
H
CD56
CD57
CD16
CD8
CD4
43%
41%
4%
1%
72%
54%
22%
11%

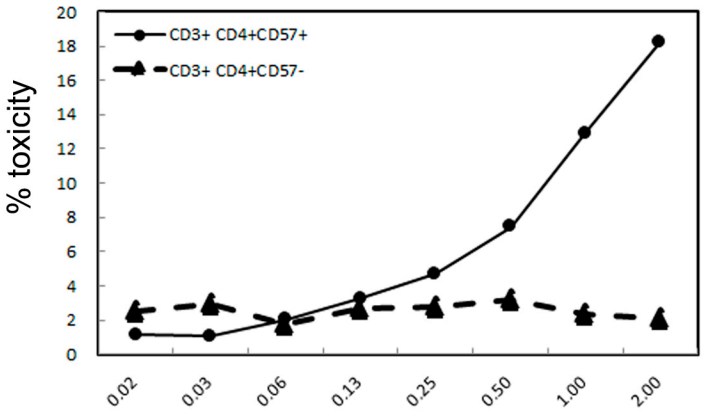

A
CD57+ Th-cells
B
CD57- Th-cells


A
CD3+ CD4+ CD57+
95%
CCR7
CD45RA
B
CD3+ CD4+ CD57+
0.034%
SSC-A
TCR Vα24-Jα18
C
CD3+ CD4- CD57+
0.017%
SSC-A
TCR Vα24-Jα18
D
CD3+ CD4+ CD57-
0.047%
SSC-A
TCR Vα24-Jα18


A
Unstimulated CD57+ Th cell
IL-4
IFN-γ
B
Unstimulated CD57+ Th cell
IL-17A
IFN-γ
C
Stimulated CD57+ Th cell
74%
IL-4
IFN-γ
D
Stimulated CD57+ Th cell
75%
IL-17A
IFN-γ



E/T
K562-A24/C II TA



E/T
K562-A24

Patients no.	Age (years)	Sex	Adverse effects	Time to lymphocytosis (months)	Peak lymphocyte count (10*9/L)
Patients developping lymphocytosis					
1	64	Male	Pleural effusion	4	3.9
2	78	Male	Pleural effusion	24	3.1
3	65	Male	Pleural effusion, CMV reactivation	8	6.2
4	45	Male	-	6	4.4
5	76	Female	Pleural effusion	9	5.9
6	62	Male	-	6	5.7
7	32	Male	-	6	4
8	51	Male	-	3	8.3
9	83	Female	-	6	9.6
10	33	Female	Skin rash	6	5
11	71	Female	Pleural effusion	6	4.9
Patients without lymphocytosis					
12	21	Female	Skin rash	-	1.6
13	39	Male	Colitis	-	1.4
14	48	Female	-	-	1.6
15	49	Male	Pleural effusion	-	2.7
16	70	Male	-	-	1.8
17	57	Male	Pleural effusion	-	2.8
18	76	Male	-	-	0.8
19	61	Female	-	-	1.3
20	55	Female	-	-	1.9
21	47	Male	Skin rash	-	1.6
22	35	Male	Skin rash	-	1.4