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Pre-diagnostic genotyping identifies T1D subjects with impaired Treg IL-2 signaling and an elevated proportion of FOXP3⁺IL-17⁺ cells

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Abstract

T-regulatory cells (Tregs) are essential for immune tolerance, and animal studies implicate their dysfunction in type 1 diabetes (T1D) pathogenesis. Tregs require interleukin-2 (IL-2) for their suppressive function, and variants in IL-2/IL-2R pathway genes have been associated with T1D. We previously reported that recent-onset T1D subjects have an increased population of FOXP3^{lo} Tregs that secrete the pro-inflammatory cytokine, interleukin-17 (IL-17). We hypothesize that IL-2 signaling defects may drive T1D development by skewing protective Tregs towards an inflammatory Th17 phenotype. Overall, we found that the proportion of FOXP3⁺IL-17⁺ cells in T1D subjects pre-diagnosis was unchanged compared with healthy controls. However, stratification by *IL2RA* single-nucleotide polymorphisms revealed that T1D subjects with the rs3118470 CC risk variant have Tregs with IL-2 signaling defects and an increased proportion of FOXP3⁺IL-17⁺ cells before diagnosis. These data suggest a potential mechanism for genetically controlled loss of Treg function via dysfunctional IL-2 signaling in T1D.

INTRODUCTION

Type 1 diabetes is an autoimmune disease whose pathogenesis involves a defect in T-cell immune tolerance to β -cell antigens. FOXP3⁺ T-regulatory (Treg) cells, characterized by

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CONFLICT OF INTEREST

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their suppressive function, are reduced in number or defective in T1D.¹ We have reported that recent-onset T1D subjects have an elevated circulating population of Th17/Treg (CD45RA⁺FOXP3^{lo}IL-17⁺) cells.² IL-17 increases the susceptibility of β cells to cytokine-mediated destruction,³ and the presence of intermediate FOXP3⁺IL-17⁺ cells along with the ability of Tregs to convert to an 'ex-Treg' IL-17 producing phenotype is a potential pathogenic mechanism for autoimmunity in T1D.⁴

The cytokine IL-2 maintains Treg suppressive function and inhibits Th17 differentiation.⁵ IL-2 induced proliferation of Tregs is facilitated by the intracellular signaling molecule, STAT5,⁵ whose phosphorylation mediates transcription of FOXP3 and CD25.⁵ The IL-2R α subunit CD25, encoded by the *IL2RA* gene on chromosome 10p15-p14, is a key component of the IL-2 high affinity receptor and constitutively expressed on Tregs.⁵ Several T1D-associated *IL2RA* haplotypes have been identified.^{6–9} T1D-associated single-nucleotide polymorphisms (SNPs) in the *IL2RA* gene that have been described include rs12722495 (previously rs41295061), rs2104286, rs1159456, rs3118470 and rs706778.^{10–13} The rs12722495 SNP is associated with alteration in allele-specific RNA levels and variation of CD25 protein expression on CD4⁺ memory T cells.¹² Moreover, healthy controls carrying the T1D-associated rs12722495 susceptible genotype (GG) have diminished IL-2 responsiveness in Tregs, as measured by phosphorylation of STAT5.^{14,15} A genotype containing the rs3118470 SNP was shown to be significantly protective in T1D-affected sibling-pair families¹⁶ and this observation has been replicated in Japanese¹⁷ and Polish¹⁸ populations. The rs3118470 SNP has also been associated with differential allele expression of *IL2RA* messenger RNA.¹⁹

We hypothesized that T1D subjects carrying T1D-associated *IL2RA* risk genotypes have Tregs with defective IL-2 signaling and reduced surface CD25 stability and expression leading to IL-17 secretion. Thus, we investigated Treg phenotype and signaling in individuals carrying the T1D-associated risk variants at rs3118470 and rs12722495 and observed that T1D subjects with the at-risk rs3118470 variant have FOXP3⁺CD4⁺ T cells that exhibit IL-2 signaling deficits and an elevated proportion of CD4⁺ T cells that co-express FOXP3 and IL-17 pre-diagnosis. We propose that IL-2 signaling deficits may cause Tregs to transition to an IL-17-secreting pathogenic phenotype.

RESULTS

FOXP3⁺IL-17⁺ cells are not increased in proportion before diagnosis of T1D

T1D subjects with recent-onset disease carry an increased population of FOXP3⁺IL-17⁺ cells (identified by the surrogate markers CD45RA⁺FOXP3^{lo}) that lack *in vitro* suppressive capacity.² On the basis of this observation, and combined with studies indicating that Tregs may convert to pathogenic T effector phenotypes,⁴ we hypothesized that before disease presentation, Tregs in T1D subjects may convert to IL-17-secreting effector cells. To determine whether Treg conversion occurs pre-diagnosis, we assessed a cohort of subjects from the NIH TrialNet Natural History Study, which followed first-degree relatives of T1D subjects for β -cell autoantibody positivity and progression to disease.²⁰

By flow cytometry, we determined the circulating proportion of CD45RA⁺FOXP3^{lo} and FOXP3⁺IL-17⁺ cells in AB⁻ controls; AB⁺ non-progressors; and pre-diagnosis T1D subjects. No significant changes were observed in either the proportion of CD45RA⁺FOXP3^{lo} cells or FOXP3⁺IL-17⁺ cells before disease presentation (Figure 1). However, considerable variability existed among individual subjects, suggesting that some individuals may possess an increased proportion of FOXP3⁺IL-17⁺ cells pre-diagnosis.

Tregs from T1D subjects homozygous for the *IL2RA* SNP rs3118470 risk genotype (CC), have reduced pSTAT5 phosphorylation upon stimulation with IL-2

IL-2 is a critical Treg growth factor necessary for maintaining their suppressive function⁵ and defects in IL-2 availability or signaling lead to reduced Treg numbers and function.⁵ Genome-wide association studies have previously reported that the *IL2RA* SNP, rs3118470, has a strong association with T1D.¹³ To determine whether the rs3118470 genotype is specifically associated with diminished IL-2 signaling, we performed phosphoflow cytometric assays on PBMC from recent-onset T1D subjects. We found that individuals who carry one or two alleles of the rs3118470 (C) risk variant have reduced IL-2 signaling in CD25⁺FOXP3⁺ Tregs, as measured by phosphorylation levels of the downstream signaling molecule STAT5 following IL-2 stimulation (Figure 2).

Surface CD25 expression on Tregs or T effector cells is unchanged in T1D subjects homozygous for the rs3118470 risk variant (CC)

The IL-2 receptor subunit CD25 is constitutively expressed on the surface of FOXP3^{hi} Tregs and IL-2-initiated signaling upregulates and maintains CD25 expression on T cells.⁵ The observation that the rs3118470 (C) variant is associated with decreased IL-2 signaling suggested that CD25 expression may be affected by this variant. Garg *et al.*¹⁴ have shown that another T1D-associated SNP, rs12722495, reduces pSTAT5 signaling in response to IL-2, as well as CD25 expression on Tregs in healthy controls. Therefore, we hypothesized that the rs3118470 risk genotype may also alter the expression of CD25 on Tregs or T effector cells in T1D subjects pre-diagnosis.

We assessed *IL2RA* genotypes at rs12722495 and rs3118470 in the pre-diagnosis T1D subjects from the TrialNet Study described in Figure 1. T1D subjects homozygous for protective genotypes (TT at rs3118470 and GG at rs12722495) were too rare to include in the analysis ($n = 1$). In T1D subjects homozygous for the rs3118470 CC risk genotype or T1D subjects carrying the TC genotype at rs3118470, no differences were seen in the expression of CD25 on CD4⁺CD127^{lo}FOXP3^{hi} Tregs or CD4⁺FOXP3⁻ T effector cells (Figure 3a and c). Similar to previous studies in healthy controls,¹⁴ we found a decrease in CD25 expression on CD4⁺CD127^{lo}FOXP3^{hi} Tregs in T1D subjects homozygous for the rs12722495 AA risk genotype, in our pre-diagnosis subjects, as compared with T1D subjects carrying the AG genotype at rs12722495 (Figure 3b). A reduction in CD25 expression on T effector cells between the different *IL2RA* SNP genotypes was also not present (Figure 3c and d). Thus, unlike rs12722495, the functional effects of the rs3118470 CC risk genotype do not involve altering CD25 expression.

FOXP3⁺IL-17⁺CD4⁺ T cells are proportionally increased in T1D subjects homozygous for the rs3118470 risk genotype (CC)

We observed that an increased proportion of FOXP3⁺IL-17⁺ cells was present in some T1D subjects before clinical onset of disease (Figure 1). We hypothesized that unique genetic polymorphisms underlie these differences. As T1D subjects homozygous for the SNP rs3118470C have reduced STAT5 phosphorylation in CD25⁺FOXP3⁺ Tregs, and IL-2 signaling is essential for Treg maintenance,²¹ we speculated that T1D subjects carrying this SNP variant may also have an increased circulating proportion of FOXP3⁺IL-17⁺ and CD45RA⁺FOXP3^{lo} (a surrogate marker for IL-17 producing FOXP3⁺ cells²²) subsets pre-diagnosis.

Lymphocyte subsets characterized by FOXP3⁺IL-17⁺ and CD45RA⁺FOXP3^{lo} expression were compared in T1D subjects homozygous or heterozygous for the rs3118470C and rs12722495A risk variants. Individuals homozygous for rs3118470C showed an increased proportion of FOXP3⁺IL-17⁺ and CD45RA⁺FOXP3^{lo} cells compared with T1D subjects who were heterozygous at this locus (Figure 4a and c). This increase in FOXP3⁺IL-17⁺ and CD45RA⁺FOXP3^{lo} cells was not seen in AB⁺ controls (Figure 5). Significant differences were not seen in the proportion of CD45RA⁺FOXP3^{lo} or FOXP3⁺IL-17⁺ cells from T1D subjects who did not carry the *IL2RA* SNP rs12722495 (Figure 4b and d). These data indicate that the rs3118470C risk genotype, but not the *IL2RA* SNP rs12722495, is associated with an increased circulating proportion of CD45RA⁺FOXP3^{lo} and FOXP3⁺IL-17⁺ cells in pre-diagnosis T1D subjects.

DISCUSSION

Intact Treg function is critical for maintaining tolerance to self, and skewing of this cell population towards a pro-inflammatory Th17 phenotype has been implicated in pathogenesis of autoimmune conditions including T1D.⁴ We show that T1D subjects who carry a risk variant polymorphism in the *IL2RA* gene have impaired IL-2 signaling in Tregs and an elevated proportion of FOXP3⁺IL-17⁺ cells prior to disease onset. This aberrant Treg phenotype was not statistically discernible when a cohort of pre-diagnosis T1D subjects was analyzed as a whole, indicating that the risk variant confers a pathogenic risk in a subset of patients. We suggest that reduced IL-2 signaling associated with the rs3118470 SNP leads to impaired maintenance of FOXP3^{hi} expression on Tregs, resulting in an abnormal IL-17 secreting phenotype. These cells represent an intermediate cell population variably described as pathogenic Tregs or protective Th17 cells in the literature.

Previous studies have shown that healthy controls homozygous for the *IL2RA* SNPs rs2104286 or rs12722495 risk genotypes have reduced IL-2 induced STAT5 phosphorylation in CD4⁺CD25⁺ cells.^{14,15} We demonstrate that Tregs from T1D subjects homozygous for the *IL2RA* SNP rs3118470 risk genotype also have reduced phosphorylation of the STAT5 signaling molecule upon IL-2 stimulation. Thus, multiple SNPs in the *IL2RA* gene may contribute to T1D pathogenesis through a defect in Treg IL-2 signaling.

The functional consequences of impaired IL-2 signaling are SNP dependent. Subjects homozygous for the rs2104286 risk genotype demonstrate reduced Treg IL-2 responsiveness

but not decreased CD25 expression on T cells.¹⁵ We observed, similarly, that the functional consequences of the homozygous rs3118470 risk genotype are not associated with CD25 expression on the surface of Treg or T effector cells. These data contradict previous studies that have suggested that a haplotype, best marked by rs3118470 increases T1D risk through diminished expression of the IL-2R.¹⁹

Consistent with previous studies in healthy controls,¹⁴ we confirmed that the rs12722495 risk genotype reduces CD25 expression on Tregs in pre-diagnosis T1D subjects. As all *IL2RA* polymorphic variants are non-coding and fall upstream of the *IL2RA* gene or intron 1, their functional effect on CD25 surface expression might be mediated by the degree of linkage disequilibrium with other coding variants, and could therefore be variable.

We also discovered that T1D subjects homozygous for rs3118470C have elevated proportions of IL-17 secreting FOXP3⁺ cells pre-diagnosis. This difference in the proportion of FOXP3⁺ IL-17⁺ cells was not observed in individuals with the *IL2RA* rs12722495 risk genotype, suggesting that this effect is specific to *IL2RA* rs3118470. Therefore the pathogenic significance of IL-2 signaling defects in Tregs may also be explained by transition to a pro-inflammatory cytokine secreting phenotype caused by an inability to maintain high FOXP3 expression.

A limitation of our study is the rarity of samples from subjects who were pre-diagnosis for T1D. Although our studies do reach statistical significance, we would propose that these data form the basis to perform larger scale studies investigating the impact of the IL-2 SNP risk genotypes in a larger population sample available from other biobanks. These results have implications for the analysis of data from current clinical trials testing the efficacy of low-dose IL-2 therapy for T1D,²³ as subjects homozygous for either the rs3118470 or the rs12722495 risk genotypes could have reduced responses to exogenous IL-2 requiring alterations in dosing. This could lead to a personalized medicine approach, whereby the genotyping of participants for T1D-associated polymorphic variants in the *IL2RA* gene may identify a subset of subjects who will respond favorably to IL-2 therapy.

MATERIALS AND METHODS

Subject recruitment

Frozen PBMCs were obtained from the TrialNet Consortium Pathway to Prevention Study.²⁰ This included 35 autoantibody positive (AB+; positive for at least one of GADA, IA-2A, mIAA or ICA) and 43 autoantibody negative (AB –) relatives of subjects with T1D and 73 subjects who developed T1D within 3 months (these 73 pre-diagnosis subjects are those included in the genotyping analysis in Figures 3–5). In addition, 25 child subjects, within 6 months of diagnosis of T1D, were recruited from BC Children's Hospital (these 25 subjects are those included in the genotyping analysis in Figure 2).

Genotyping

The *IL2RA* SNPs rs3118470 and rs12722495 were genotyped using commercially available or custom TaqMan assays (C___1882662_10; Life Technologies, Carlsbad, CA, USA). SNPs were acceptable for analysis if they had call rates >95%, frequencies, did not deviate

from Hardy–Weinberg equilibrium ($P>0.05$), and no Mendelian errors were observed in the available complete trios. Genotype call rates for both SNPs were 98%.

Flow cytometry

Phosphoflow was performed by adapting a previously published protocol.²⁴ PBMCs were activated with or without IL-2 (Proleukin) 200 U ml⁻¹ for 10 min, fixed with Phosflow Buffer I, and permeabilized using Phosflow Buffer III before staining with anti-pSTAT5(47/Stat5Y694) and anti-CD4(RPA-T4); all from BD Biosciences, San Jose, CA, USA and anti-FOXP3(150D/E4) and anti-CD25(BC96) from eBioscience, San Diego, CA, USA. Changes in pSTAT5(Y694) mean fluorescence intensity were calculated as the difference in the geometric mean fluorescence intensity of the IL-2 stimulated sample and non-stimulated paired sample.

PBMCs were also stained with a combination of antibodies comprising anti-CD4(RPA-T4), anti-CD14(MϕP9.1), anti-CD8(RPA-T8) and anti-CD127 (hIL-7R-M21) (all from BD Biosciences); anti-CD45RA(HI100) and anti-CD25 (BC96) (all from eBioscience). The PBMC samples were suspended in R10 media with PMA (100 ng ml⁻¹), ionomycin (1 µg ml⁻¹) and Brefeldin A (10 µg ml⁻¹; all from Sigma-Aldrich, St Louis, MO, USA) for 4 h before intracellular staining. For intracellular staining, the cells were treated with perm/fix buffer (eBioscience) and stained with anti-FOXP3 (259/C7, BD Biosciences) and anti-IL17A(eBio64DEC17). The data were acquired using a FACS-LSRII (BD Biosciences) and analyzed using FlowJo (TreeStar, Ashland, OR, USA). Examples of gating strategies are shown in Figure 6. The unpaired Student's *t*-test or one-way analysis of variance test was used for statistical comparisons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

FOXP3⁺IL-17⁺ cells are not increased in proportion before diagnosis of T1D. Percentage of CD4⁺ T cells that are (a) CD45RA⁻FOXP3^{lo} and (b) IL-17⁺FOXP3⁺ in PBMCs from AB⁻ controls, AB⁺ non-progressors and T1D before diagnosis. (b). PBMCs were unstimulated in a. PBMCs were stimulated with PMA/Ionomycin in the presence of Brefeldin A in b. No significant differences between any cohort were observed (CD45RA⁻FOXP3^{lo}: AB⁻ controls $2.4 \pm 0.12\%$ vs AB⁺ non-progressors $2.1 \pm 0.185\%$ vs T1D before diagnosis $1.9 \pm 0.1\%$; one-way analysis of variance (ANOVA) $P = 0.06$; FOXP3⁺IL-17⁺: AB⁻ controls $0.15 \pm 0.02\%$ vs AB⁺ non-progressors $0.13 \pm 0.08\%$ vs T1D before diagnosis $0.11 \pm 0.01\%$; one-way ANOVA $P = 0.1$).

Figure 2.

Tregs from T1D subjects homozygous for the *IL2RA* SNP rs3118470 risk genotype (CC) have reduced pSTAT5 phosphorylation upon stimulation with IL-2. STAT5 phosphorylation in FOXP3⁺CD25⁺ T cells following IL-2 stimulation from T1D subjects with the CC, TC and TT genotype at rs3118470. PBMCs were stimulated with or without the presence of 200 IU ml⁻¹ of IL-2 for 10 min. The cells were fixed/permeabilized and phosphoflow staining performed. Analysis of change in mean fluorescence intensity (MFI) of pSTAT5 upon IL-2 stimulation was calculated by gating on CD4⁺FOXP3⁺CD25⁺ Treg cells and measuring the change in pSTAT5 MFI between cells stimulated with and without IL-2. FOXP3⁺CD25⁺ Tregs from T1D subjects homozygous for the rs3118470C allele, had reduced STAT5 phosphorylation upon IL-2 stimulation, compared with subjects with the TC or TT genotypes (MFI pSTAT5: CC = 184.6 ± 41.80 vs TC = 334.5 ± 46.83 vs TT = 438 ± 91.97, one-way analysis of variance (ANOVA), *P* = 0.018) (a). T1D-associated risk genotypes (*IL2RA* SNP rs3118470C) is shown in gray. A representative plot of pSTAT5 levels after 30 min of IL-2 stimulation of T1D PBMC gated on CD4⁺FOXP3⁺CD25⁺ cells is shown (b) with the y axis being scaled so the mode represents 100%. Red = homozygous for risk genotype, Green = heterozygous at the rs3118470 locus, Blue = homozygous for the non-risk genotype and Gray = background levels with no IL-2 staining. ***P* < 0.05.

Figure 3.

Surface CD25 expression on Tregs or T effector cells is unchanged in T1D subjects homozygous for the rs3118470 risk variant (CC). The mean fluorescence intensity (MFI) of CD25 on CD4⁺CD127^{lo}FOXP3^{hi} Treg cells (**a** and **b**) or CD4⁺FOXP3⁻ T effector cells (**c** and **d**) in PBMCs of T1D subjects with different genotypes at rs3118470 (**a** and **c**) and rs12722495 (**b** and **d**). T1D-associated risk genotypes rs3118470 (**c**) and rs12722495 (**a**) are shown in gray. PBMCs were stained using flow cytometry techniques, median fluorescence intensity of CD25 was assessed on gated CD4⁺CD127^{lo}FOXP3^{hi} Treg cells or CD4⁺FOXP3⁻ T effector cells. The rs3118470 risk genotype (CC) does not alter CD25 expression on CD4⁺CD127^{lo}FOXP3^{hi} Treg cells (**a**) or CD4⁺FOXP3⁻ T effector cells (**c**). (rs3118470: CD25 MFI on CD127^{lo}FOXP3⁺: CC = 309 ± 26 vs TC = 267 ± 23, unpaired *t*-test, *P* = 0.23; CD25 MFI on CD4⁺FOXP3⁻: CC = 39 ± 2.2 vs TC = 33 ± 2.1, unpaired *t*-test, *P* = 0.08). However, T1D subjects homozygous for the rs12722495 risk genotype (AA) have a reduced expression of CD25 on the surface of Treg cells (rs12722495: CD25 MFI on CD127^{lo}FOXP3⁺: AA = 263 ± 16 vs AG = 364 ± 40, unpaired *t*-test *P* = 0.007; (**b**) but no difference in CD25 expression on the surface of Teff cells (rs12722495 CD25 MFI on CD4⁺FOXP3⁻: AA = 37 ± 2.5 vs AG = 43 ± 6.7, unpaired *t*-test, *P* = 0.3; (**d**) compared with a single copy of rs12722495 risk allele. ***P* < 0.05.

Figure 4.

FOXP3⁺IL-17⁺CD4⁺ T cells are proportionally increased in T1D subjects homozygous for the rs3118470 risk genotype (CC). Percentage of CD4⁺ cells that are CD45RA⁻FOXP3^{lo} (a and b) or FOXP3⁺IL-17⁺ (c and d) in PBMCs of T1D subjects before diagnosis with one or two copies of the rs3118470 risk allele (a and c) or T1D subjects before diagnosis with one or two copies of the rs12722495 risk allele (b and d). PBMCs were stained using flow cytometry techniques to identify the CD45RA⁻FOXP3^{lo} and FOXP3⁺IL-17⁺ subsets. CD4⁺ CD45RA⁻FOXP3^{lo} T cells and FOXP3⁺IL-17⁺ cells in the PBMCs of T1D subjects homozygous for the rs3118470 risk genotype (CC) were elevated in proportion compared to individuals heterozygous at this locus (T1D subjects: CD45RA⁻FOXP3^{lo}: CC = 2.02 ± 0.12% vs TC = 1.59 ± 0.12%, unpaired *t*-test *P* = 0.014; FOXP3⁺IL-17⁺: CC = 0.15 ± 0.02% vs TC = 0.06 ± 0.007%, *P* = 0.002; a and c). This difference was not observed between homozygous and heterozygous individuals at the rs12722495 variant (CD45RA⁻FOXP3^{lo}: AA = 1.9 ± 0.14% vs AG = 1.9 ± 0.17%, unpaired *t*-test *P* = 0.98; FOXP3⁺IL-17⁺: AA = 0.11 ± 0.02% vs AG = 0.10 ± 0.01%, unpaired *t*-test, *P* = 0.82; b and d). T1D-associated risk genotypes rs3118470 (CC) and rs12722495 (AA) are shown in gray. ***P* < 0.05.

Figure 5.

There is no difference in proportion of FOXP3⁺IL-17⁺ cells in controls with different genotypes at *IL2RA* rs3118470. Percentage of CD4⁺ cells that are CD45RA⁻FOXP3^{lo} (a) or FOXP3⁺IL-17⁺ (b) in PBMCs from AB⁻ controls carrying one or two copies of the *IL2RA* SNP rs3118470 risk variant. PBMCs were stained using flow cytometry techniques to identify the CD45RA⁻FOXP3^{lo} subset and FOXP3⁺IL-17⁺ subset. There is no significant difference observed (AB⁻controls: CD45RA⁻FOXP3^{lo}: CC $2.7 \pm 0.2\%$ vs TC $2.4 \pm 0.16\%$, unpaired *t*-test, $P = 0.24$; FOXP3⁺IL-17⁺: CC $0.19 \pm 0.03\%$ vs TC $0.14 \pm 0.03\%$, unpaired *t*-test $P = 0.18$).

Figure 6.

Flow cytometry panel gating strategy for Treg immunophenotyping. All the samples were initially gated on lymphocytes and CD4⁺ CD14⁺ cells. Gating strategies for (a) CD45RA⁻FOXP3^{lo} and (b) FOXP3⁺IL-17⁺ (c and d) CD25 MFI on T effector and Treg cell subsets, are shown. MFI, mean fluorescence intensity.