Global Characterization of Differential Gene Expression Profiles in Mouse $\gamma^1+$ and $\gamma^4+$ $\delta$ T Cells

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Abstract

Peripheral $\gamma^\delta$ T cells in mice are classified into two major subpopulations, $\gamma^1+$ and $\gamma^4+$, based on the composition of T cell receptors. However, their intrinsic differences remain unclear. In this study, we analyzed gene expression profiles of the two subsets using Illumina HiSeq 2000 Sequencer. We identified 1995 transcripts related to the activation of $\gamma^1+$ $\gamma^\delta$ T cells, and 2158 transcripts related to the activation of $\gamma^4+$ $\gamma^\delta$ T cells. We identified 24 transcripts differentially expressed between the two subsets in resting condition, and 20 after PMA/ionomycin treatment. We found that both cell types maintained phenotypes producing IFN-$\gamma$, TNF-$\alpha$, TGF-$\beta$ and IL-10. However, $\gamma^1+$ $\gamma^\delta$ T cells produced more Th2 type cytokines, such as IL-4 and IL-5, while $\gamma^4+$ $\gamma^\delta$ T cells preferentially produced IL-17. Our study provides a comprehensive gene expression profile of mouse peripheral $\gamma^1+$ and $\gamma^4+$ $\gamma^\delta$ T cells that describes the inherent differences between them.

Introduction

$\gamma^\delta$ T cells were discovered more than 30 years ago. Although considerable progress has been made in characterizing their biological significance, much remains unknown. $\gamma^\delta$ T cells arise earlier than $\beta^\delta$ T cells during thymic ontogeny, predominantly at the early stage of fetal development [1]. After birth, however, $\gamma^\delta$ T cells make up a minor fraction of circulating T lymphocytes in rodents and humans. Similar to $\beta^\delta$ T cells, $\gamma^\delta$ T cells also have a diverse repertoire of T cell receptors (TCR) derived through somatic rearrangement of V, D and J gene segments. Although few V, D and J gene elements are responsible for genetic rearrangement, additional diversity is added to the $\gamma$ and $\delta$ chains via junctional diversification processes [2].

$\gamma^\delta$ T cells exert diverse functions, however, individual subsets within the population appear to be biased toward specialized functions [1]. Mouse peripheral lymphoid $\gamma^\delta$ T cells are classified into two major subsets, $\gamma^1+$ and $\gamma^4+$ $\gamma^\delta$ T cells, depending on their TCR expression [1,3,4]. $\gamma^1+$ and $\gamma^4+$ $\gamma^\delta$ T cells perform distinct functions in many disease models. For example, $\gamma^1+$ $\gamma^\delta$ T cells produce IL-4 and IFN-$\gamma$ in the liver [5], and $\gamma^4+$ $\gamma^\delta$ T cells produce IFN-$\gamma$ or IL-17 depending on the studied models [6]. $\gamma^1+$ and $\gamma^4+$ $\gamma^\delta$ T cells function as oppositional pairs in diseases including coxsackievirus B3 infection [7], West Nile virus infection [4], airway hyperresponsiveness [8,9], macrophage homeostasis [10] and ovalbumin induced IgE production [11]. However, the functional relatedness of $\gamma^1+$ and $\gamma^4+$ $\gamma^\delta$ T cells remains unresolved, partly due to a lack of comprehensive analysis and comparison of gene expression. Although, gene-expression profiles of emergent $\gamma^\delta$ TCR+ thymocytes have been reported [12], a comprehensive analysis of peripheral $\gamma^1+$ and $\gamma^4+$ $\gamma^\delta$ T cells functional differences has not been reported. This is likely due to the limited number of cells that can be obtained from healthy mice.

In this study, we expanded $\gamma^1+$ and $\gamma^4+$ $\gamma^\delta$ T cells simultaneously from the same pool of mouse splenocytes. We comprehensively analyzed gene expression profiles using Illumina’s sequencing technology. We identified 1995 transcripts related to the activation of $\gamma^1+$ $\gamma^\delta$ T cells, and 2158 transcripts were related to the activation of $\gamma^4+$ $\gamma^\delta$ T cells. Interestingly, only 24 transcripts were differentially expressed between two subsets in resting condition, and 20 transcripts after PMA/ionomycin-induced activation. Both cells produced high levels of IFN-$\gamma$, TNF-$\alpha$, TGF-$\beta$ and IL-10. However, $\gamma^1+$ $\gamma^\delta$ T cells produced more Th2 type cytokines, while $\gamma^4+$ $\gamma^\delta$ T cells tended to produce more IL-17. These findings describe the inherent differences between $\gamma^1+$ and $\gamma^4+$ $\gamma^\delta$ T cells.

Materials and Methods

Mice

Male C57BL/6j mice aged 6–8 weeks were purchased from the National Institute for Food and Drug Control. All mice were maintained under specific pathogen-free conditions in the Experimental Animal Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. All animal experiments were approved by and performed in accordance with the

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. The raw data files have been deposited in NCBI’s Sequence Read Archive (SRA) and are accessible through SRA Series accession number SRP042029.

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guidelines of the International Agency for Research on Cancer’s Animal Care and Use Committee and IBMS/PUMC’s Animal Care and Use Committee.

Expansion of V_γ1^+ and V_γ4^+ γδ T cells

V_γ1^+ and V_γ4^+ γδ T cells were expanded from splenocytes as described previously [13]. Briefly, flat-bottom 24 well plates were coated with 500 µl purified anti-mouse TCR γδ antibody (UC7–13D5, 1 µg/ml; Biolegend) at 37°C for 2 hours. Splenocytes were collected from six male C57BL/6J mice to decrease individual variation. Erythrocytes were lysed in Tris-NH4Cl buffer. Cells were then loaded onto a sterile nylon wool column, sealed and incubated at 37°C with 5% CO2 for 45 minutes. 5 × 10^6 cells were eluted and added to the Ab-coated wells (4 × 10^5 cells/well) and cultured in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal calf serum and IL-2 (200 IU/ml). After 8 days of expansion, the proportion of γδ T cells reached approximately 90% as determined by Flow Cytometry.

Cell sorting and stimulation

1.0 × 10^6 V_γ1^+ and 1.2 × 10^6 V_γ4^+ γδ T cells were sorted by Flow Cytometric Cell Sorting (FACS) with PE conjugated anti-mouse TCR V_γ1.1/Gr4 antibody (2.11, Biolegend) and APC conjugated anti-mouse TCR V_γ2 antibody (UC3–10A6, Biolegend). The purity of sorted cells was more than 99%. 5 × 10^6 cells were eluted and added to the Ab-coated wells (4 × 10^5 cells/well) and cultured in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal calf serum and IL-2 (200 IU/ml). After 8 days of expansion, the proportion of γδ T cells reached approximately 90% as determined by Flow Cytometry.

Processing samples for Illumina sequencing

We prepared the Illumina libraries according to the manufacturer’s instructions. Briefly, mRNAs were extracted from total RNA by mRNA enrichment kit (Life technologies, USA) followed by fragmentation of mRNA into 250–350 bp sizes. The first strand cDNAs were synthesized using DNA Polymerase I followed by the addition of a single A base at the ends for the ligation to the adapters. After purification, the final cDNA library was created by PCR. Finally, 400–500 bp products were used for cluster generation, 36 bp single-end sequencing was performed using Illumina HiSeq 2000 Sequencer according to the manufacturer’s instructions (Beijing Berry Genomics Co. Ltd. China). The RNA-Seq raw data files have been deposited in NCBI’s Sequence Read Archive (SRA) and are accessible through SRA Series accession number SRP042029.

Analysis of RNA-seq data

We performed base calling using CASAVA 1.7 software (Illumina). Low quality and polluted adapter reads were filtered; clean reads were stored on fastq files. The sequence reads were aligned to the mouse genome (mm9), and gene expression was calculated by RPKM value. Differentially expressed transcripts were identified using General Chi-square test analysis. Q values were obtained by the “BH” method [14]. NIH DAVID web server was used for the functional annotation clustering analysis of differentially expressed transcripts.

Quantitative RT-PCR

Several genes from V_γ1^+ and V_γ4^+ γδ T cells were selected for verification from biological replicates with real-time quantitative PCR. RNA was extracted as described above. 500 ng of total RNA was reverse transcribed using PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio). Gene-specific primers are listed in (Table 1). The real-time quantitative PCR was performed on the StepOnePlus Real-Time PCR System (Life Technologies) using SYBR green labeling (SYBR Premix Ex Taq II; Takara Bio). A cycle threshold (Ct) was assigned at the beginning of the logarithmic phase of PCR amplification and relative quantitation was done using the 2^-ΔΔCt method. β-actin was used for normalization control.

Cytokines

Cells were stimulated 4 h with PBS or PMA and Ionomycin then pelleted by centrifugation. Determined cytokine concentration in cell-free supernatants by enzyme linked immunosorbent assay (ELISA; R&D Systems) as described previously [15] and MILLIPLEX MAP Mouse Cytokine Kit (MT17MAG47)

| Table 1. Gene-specific primers for real-time quantitative PCR. |
|-------------|-----------------|-----------------|
| Specificity | Primer orientation | Sequence (5’ → 3’) |
| IL-4        | Forward          | ACCGAGATGGATGTCCTCCAAC |
|             | Reverse          | AGCACCTTTGAAGCCCTACAGA |
| IL-5        | Forward          | TGGACCTTCTGGTCTCTACTATGA |
|             | Reverse          | TTGGAAATGACATCTCCACAGTC |
| IL-17A      | Forward          | CTCGATCGACGCGCAAC |
|             | Reverse          | TCGGTGCCATCTACATGA |
| IL-17F      | Forward          | ATGAAAGTGCCGACGAAAC |
|             | Reverse          | ATCGACGATGGCACTCCCAAC |
| SCART 2     | Forward          | GGATCCAGGGCCCTTGTGA |
|             | Reverse          | TGGCATGACGCCGACGAAAC |
| beta-actin  | Forward          | CATCGTAAAGAGACCTATGCGCAAC |
|             | Reverse          | ATGGAGCCACCGATCCCA |

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Figure 1. Vγ1+ and Vγ4+ γδ T cells are the major subpopulations in the spleen. (A) γδ T cells account for approximately 1.5% of total splenocytes. Isolated fresh Vγ1+ and Vγ4+ γδ T cells comprised approximately 35% and 25% of γδ T cells respectively. (B) 1.5 × 10^8 cells were expanded simultaneously from a single pool of mouse splenocytes with purified pan anti-mouse TCR γδ antibody (UC7–13D5). After 8 days, Vγ1+ and Vγ4+ γδ T cells comprised approximately 40% and 30% of the expanded cells, respectively. 1.0 × 10^7 Vγ1+ γδ T cells were sorted by FACS with PE conjugated anti-mouse TCR Vγ1.1/Cr4 antibody and 1.2 × 10^7 Vγ4+ γδ T cells were sorted by FACS with APC conjugated anti-mouse TCR Vγ2 antibody. Purity of sorted cells was >99%. Data are representative of four independent experiments.

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Results Expansion and isolation of Vγ1+ and Vγ4+ γδ T cells from mouse splenocytes γδ T cells account for approximately 1–2% of total splenocytes in healthy mice and Vγ1+ and Vγ4+ γδ T cells comprised approximately 35% and 25% respectively (Figure 1A). Therefore, we expanded the cells from mouse spleens in vitro for RNA-seq analysis. Although Vγ1+ and Vγ4+ γδ T cells can be expanded separately with sorted splenic γδ T cells using anti-Vγ1 and anti-Vγ4 Abs [16,17], potentially important biological interactions between the subsets during culture would be neglected. We therefore established a primary culture method to expand the cells simultaneously from the same pool of mouse splenocytes with pan anti-mouse TCR γδ antibodies (UC7–13D5) and IL-2. After 8 days of expansion, the proportion of γδ T cells reached approximately 80%, Vγ1+ and Vγ4+ γδ T cells comprised approximately 40% and 30% of the expanded cells, respectively (Figure 1B). No significant change was observed in the ratio of γ1 cells to γ4 cells in the in vitro expanded γδ T cells when compared with that of freshly isolated γδ T cells (in vivo subsets). γδ T cells were not screwed to one preferential subset after in vitro expansion, suggesting that in vitro expanded γδ T cells with anti-mouse TCR γδ antibodies plus IL-2 were still representative of in vivo subsets of γδ T cells. Expanded Vγ1+ and Vγ4+ γδ T cells were then sorted by FACS with PE-conjugated anti-mouse TCR Vγ1.1/Cr4 antibody and APC conjugated anti-mouse TCR Vγ2 antibody. We found the purities of sorted Vγ1+ and Vγ4+ γδ T cells were more than 99% (Figure 1B).

cDNA library preparation for RNA sequencing from resting and activated Vγ1+ and Vγ4+ γδ T cells

In order to compare gene expression profiles between subsets in both the resting and activated state, sorted cells were rested overnight at 37°C then stimulated 4 h with either PBS (control) or 20 ng/ml of PMA+0.5 μg/ml of Ionomycin (activated) before mRNA extraction and fragmentation. After cDNA synthesis, adapter ligation and PCR amplification, four cDNA libraries were constructed for the resting and activated γδ T cell subsets. 400–500 bp-sized products were used for cluster generation and 36 bp single-end sequencing was performed by using Illumina HiSeq 2000 Sequencer. Approximately 28 million clean reads were obtained from each sample. More than 88% of reads were mapped to the mouse genome using the default setting in TopHat, suggesting high quality of RNA-seq (Table 2). Cufflinks with default settings were used to assemble the mapped reads against the ENSEMBL gene structure annotation, and estimated expression levels for each transcript. More than 18,286 genes were detected. 25.4–26.1% of genes showed expression levels changed by at least four fold while the majority of genes changed less than four fold (Figure 2, Dataset S1).

Differential gene expression between Vγ1+ and Vγ4+ γδ T cells

RNA-seq results show Vγ1+ and Vγ4+ γδ T cells share similar transcript profiles in both the resting and activated subsets. We identified 24 transcripts with differential expression between the
resting $\gamma^1^+$ and $\gamma^4^+$ $\gamma\delta$ T cells (Table 3). We used the Database for Annotation, Visualization and Integrated Discovery (DAVID), an on-line functional annotation tool for gene enrichment analysis, to gain further insight into biological pathways associated with the differentially expressed gene transcripts. We found most of the differentially expressed genes in the resting subsets related to chemokines, transcription and the plasma membrane (Table 3). Resting $\gamma^1^+$ $\gamma\delta$ T cells expressed higher levels of XCL1 and CCL1 compared with $\gamma^4^+$ $\gamma\delta$ T cells, suggesting $\gamma^1^+$ $\gamma\delta$ T cells possess higher chemotactic activity for lymphocytes and monocytes. $\gamma^4^+$ $\gamma\delta$ T cells displayed higher levels of Rore, Sox13 and Scart2 expression. In addition, high levels of Bclaf1 and Atf2 were expressed in $\gamma^4^+$ $\gamma\delta$ T cells while Arnt2, Hmga1 and Zfp386 were preferentially expressed in $\gamma^1^+$ $\gamma\delta$ T cells.

In the PMA/Ionomycin-activated $\gamma^1^+$ and $\gamma^4^+$ $\gamma\delta$ T cells, we found 20 differentially expressed genes, most of which are related to cytokines, cell differentiation, transcription and translation (Table 3). Activated $\gamma^1^+$ $\gamma\delta$ T cells expressed higher levels of IL-4 and IL-5. $\gamma^4^+$ $\gamma\delta$ T cells secreted more IL-17A and IL-17F. Alternatively spliced transcript variants Smurf1, Ppiln1, Ilf3 and Sema6d were preferentially expressed in $\gamma^4^+$ $\gamma\delta$ T cells. $\gamma^1^+$ $\gamma\delta$ T cells preferentially expressed Bcl11b, Hmga1 and a second spliced transcript variant of Sema6d. These results taken together indicate that a very small number of genes are sufficient to define the characteristics of these two subsets of $\gamma\delta$ T cells.

Validation of differentially expressed genes in $\gamma^1^+$ and $\gamma^4^+$ $\gamma\delta$ T cells

We measured expression levels in both subsets by PCR to verify whether the genes identified via RNA-sequencing were differentially expressed in $\gamma^1^+$ and $\gamma^4^+$ $\gamma\delta$ T cells. Several genes from both subsets were randomly selected for verification (Figure 3). Consistent with the RNA-seq results, Sema6d mRNA was only detectable in $\gamma^4^+$ $\gamma\delta$ T cells (Figure 3E). Real-time quantitative PCR confirmed that expression levels of IL-4 and IL-5 mRNA were significantly higher in activated $\gamma^1^+$ $\gamma\delta$ T cells compared with activated $\gamma^4^+$ $\gamma\delta$ T cells (Figure 3A and 3B). Inactivated and $\gamma^4^+$ $\gamma\delta$ T cells (Figure 3C and 3D). ELISA results confirmed that IL-4 was mainly expressed in activated $\gamma^1^+$ $\gamma\delta$ T cells whereas IL-17 was predominantly expressed in activated $\gamma^4^+$ $\gamma\delta$ T cells (Figure 4A and 4B). Together, all of the genes randomly selected for expression

Table 3. 24 transcripts expressed differently between resting $\gamma^1^+$ and $\gamma^4^+$ $\gamma\delta$ T cells.

| Category          | GN     | AN          | $\gamma^1$ RPKM | $\gamma^4$ RPKM | Gene function                                      |
|-------------------|--------|-------------|-----------------|-----------------|---------------------------------------------------|
| Chemokine         | XCL1   | NM_008510   | 340.582         | 59.158          | Chemotactic activity                              |
|                   | CCL1   | NM_011329   | 84.8039         | 11.7718         | Chemotactic activity                              |
| Transcription     | *BCLAF1| NM_001025392| 0.10265         | 8.85303         | Transcriptional repressor                        |
|                   | RORC   | NM_011281   | 0.0844492       | 0.761408        | Orphan nuclear receptor                           |
|                   | SOX13  | NM_011439   | 0.909818        | 4.99875         | Transcription factor                              |
|                   | *ATF2  | NM_009715   | 0.702722        | 4.69872         | Transcriptional activator                         |
|                   | *ARNT2 | NM_007488   | 0.799234        | 0.135113        | Recognizes xenobiotic response element (XRE)      |
|                   | *HMGAl | NM_00139356 | 5.3784          | 0.84425         | Regulation of inducible gene transcription        |
|                   | *ZFP386| NM_019565   | 20.4163         | 0.927052        | Transcriptional regulation                        |
| Plasma membrane   | *CD74  | NM_001042605| 3.99813         | 0.540024        | Antigen processing                                |
|                   | *CTC1  | NM_00113256 | 0.062176        | 6.89298         | Uncharacterized                                   |
|                   | *ABI1  | NM_145994   | 0.0359843       | 6.83637         | Cytoskeletal reorganization and EGFR signaling    |
|                   | *CACNB3| NM_001044741| 0.410331        | 4.17596         | The beta subunit of calcium channels              |
|                   | *SYT13 | NM_183396   | 1.88579         | 0.141228        | Vesicle trafficking                               |
|                   | *SLC17A| NM_080853   | 0.80135         | 0.0264333       | Mediates the uptake of glutamate                 |
|                   | *TMEM219| NM_028389   | 0.071877        | 43.5973         | Unknown                                          |
| Miscellaneous     | SCART2 | NM_175533   | 0.22478         | 3.02098         | Scavenger receptor                                |
|                   | *SENP7 | NM_00103972 | 0.178749        | 7.79658         | Protease                                         |
|                   | *ENTPD5| NM_007647   | 8.35339         | 0.00516434      | Promote reglycosylation                           |
|                   | *FAR1  | NM_026143   | 0.900286        | 6.64512         | Fatty Acyl CoA Reductase 1                        |
|                   | *GOLGA2| NM_133852   | 0.942965        | 7.61176         | Maintaining cis-Golgi structure                   |
|                   | *ITIH5 | NM_172471   | 2.98889         | 0.530312        | Tumor suppressor                                  |
|                   | *PPHNL1| NM_001083114| 4.76635         | 0.114948        | Epidermal integrity and barrier formation        |
|                   | *BC003331| NM_001077237| 5.03977         | 0.527241        | LAGI-Interacting Protein                         |

GN, Gene name; AN, Accession Number; $\gamma^1$ RPKM, the RPKM value of gene in resting $\gamma^1^+$ $\gamma\delta$ T cells; $\gamma^4$ RPKM, the RPKM value of gene in resting $\gamma^4^+$ $\gamma\delta$ T cells; *", Gene's alternatively spliced transcript variants.
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analysis were consistent with RNA-seq results, confirming differential expression in Vγ1+ and Vγ4+ γδ T cells.

**Gene expression in the resting compared with PMA/ Ionomycin-activated state**

PMA/Ionomycin treatment induces a robust non-TCR mediated response in γδ T cells [18]. As expected, we found both Vγ1+ and Vγ4+ γδ T cells responded robustly to PMA/Ionomycin treatment, as reflected in the total number of genes that significantly changed in each subset. 1,995 transcripts were differentially expressed between the resting and activated Vγ1+ γδ T cells, with 560 up-regulated and 1,435 down-regulated genes (q<0.05) (Figure 5A, Dataset S2). 2,158 transcripts were differentially expressed between resting and activated Vγ4+ γδ T cells, with 622 up-regulated and 1,536 down-regulated genes (q<0.05) (Figure 5A, Dataset S3). For a global perspective on gene dynamics, two heat maps of the 1,995 and 2,158 differentially expressed gene transcripts were generated using hierarchical clustering analysis (Figure 5B).

DAVID functional annotation clustering analysis showed the 1,995 transcripts identified via activation of Vγ1+ γδ T cells were enriched for 32 KEGG pathways (p<0.05) (Table 5). 2,158 transcripts identified via activation of Vγ4+ γδ T cells were enriched for 29 KEGG pathways (p<0.05) (Table 6). Our comparison of the KEGG pathways between the two subsets showed they share most of the same signal pathways including cytokine-cytokine receptor interaction, Jak-STAT signaling pathway, hematopoietic cell lineage, apoptosis, and pathways in cancer. Interestingly, both Vγ1+ and Vγ4+ γδ T cells showed connections to the intestinal immune network for IgA production, biosynthesis of unsaturated fatty acids, glycosphingolipid biosynthesis, glutathione metabolism, and purine and pyrimidine metabolism.

We analyzed the expression levels of some common representative markers in resting Vγ1+ and Vγ4+ γδ T cells (Table 7). Both subsets expressed high levels of the β and γ chains in the cytokine receptor genes IL-2R, IL-7R and interferon gamma receptor 1. We measured medium expression levels of interferon (alpha and beta) receptor 1 and 2, α and β chains of IL-10R, IL-18 receptor 1,
IL-18 receptor beta, IL-21R, α chain of IL-27R, beta receptor II of transforming growth factor and IL-4R. Both Vγ1+ and Vγ4+ γδ T cells expressed high levels of TGF-β, known to down-regulate immune response and a key regulator of T cell and Th17 differentiation [19–21]. Additionally, both Vγ1+ and Vγ4+ γδ T cells expressed IL-16. In contrast, IFN-γ, TNFα and LTA were expressed at relatively low levels during the resting condition. Several conventional T cell surface antigens were highly expressed in Vγ1+ and Vγ4+ γδ T cells, including CD2, CD3, CD7, CD27, CD37, CD47, CD48, CD52, CD53, CD82 and CD97. However, some surface markers, including CD25, CD44, and CD69 were expressed at low levels.

Resting Vγ1+ and Vγ4+ γδ T cells expressed high levels of Fas ligand and the granzymes Gzma and Gzmb. NK cells associated receptors including NKG2A, CD94 and NKG2D were also highly expressed by both resting subsets (Table 7). Interestingly, several integrins were highly expressed including Ighb7 (Ly69), Ighb2 (Cd18), Igal (Cd11a), Igae (Cd103) and Ighb1 (Cd29) (Table 7). However, none of the TLRs showed high expression levels in either subset. In fact, TLR1, TLR6 and TLR12 were the only three detected, and with very low expression levels.

PMA/Ionomycin treatment activates Vγ1+ and Vγ4+ γδ T cells, upregulating T cell activation markers CD25, CD69 and CD44 along with several cytokines. Therefore, we analyzed the expression of these representative markers in activated Vγ1+ and Vγ4+ γδ T cells. As expected, PMA/Ionomycin treatment induced expression of XCL1, CCL3, CCL4, CCL1, IFN-γ, Lta, Csf2, TNF-α, IL-2, Gzmb and Gzmc (Table 8). MILLIPLEX results further confirmed that both Vγ1+ and Vγ4+ γδ T cells produced high levels of TNF-α, IL-2 and IFN-γ after PMA/Ionomycin treatment (Figure 4C, 4D and 4E). This is consistent with the hypothesis that γδ T cells acquire a pre-activated status poised to actively transcribe genes related to effector functions. Interestingly, IL-10, a Th1 cytokine down-regulator, was also highly expressed by both Vγ1+ and Vγ4+ γδ T cells (Table 8).

We analyzed the expression levels of transcription factors related to Th cell differentiation and cytokine secretion (Dataset S4). Both Vγ1+ and Vγ4+ γδ T cells expressed high levels of Gata3, T-bet, Eomes, Foxp1, Stat1, Stat3, Stat4, Stat5a, Stat5b, Stat6, Runx3, Ifi1, Ikbj1, Ikbj3, Ets1, Junb and Batf at resting condition. After PMA/Ionomycin treatment, the expression levels of Stat5a and If4 were upregulated significantly. The expression levels of T-bet, Eomes, Foxp1, Stat5b, Gfi1 and Junb were

Figure 3. Gene verification with real-time quantitative PCR. Several genes from Vγ1+ and Vγ4+ γδ T cells were selected for verification against biological replicates using real-time quantitative PCR (A-E). Expression data for each gene were normalized against β-actin. Data shown are the means ± SD (error bars). (*p<0.05, **p<0.01, ***p<0.001, unpaired two-tailed Student’s t-test). Data are representative of three independent experiments. doi:10.1371/journal.pone.0112964.g003
Figure 4. Cytokine expression. ELISA results of (A) IL-4 and (B) IL-17 after PBS or PMA and Ionomycin treatment. MILLIPLEX results of (C) TNF-α, (D) IL-2 and (E) IFN-γ after PBS or PMA and Ionomycin treatment. Data shown are mean ± SD (error bars). (* p≤0.05, ** p≤0.01, *** p≤0.001, unpaired two-tailed Student’s t-test). Data are representative of three independent experiments. doi:10.1371/journal.pone.0112964.g004

Figure 5. Changes in gene expression profile among Vγ1+ and Vγ4+ γδ T cells. (A) The number of up and down regulated genes between resting and activated Vγ1+ and Vγ4+ γδ T cells. (B) Heat maps of 1,995 (Vγ1+) and 2,158 (Vγ4+) differentially expressed transcripts associated with activated cells using hierarchical clustering analysis. γ1 vs γ1 T, resting Vγ1+ vs activated Vγ1+ γδ T cells; γ4 vs γ4T, resting Vγ4+ vs activated Vγ4+ γδ T cells; RPKM, Reads Per Kilo bases per Million reads. doi:10.1371/journal.pone.0112964.g005
upregulated slightly. Interestingly, the expression levels of Gata3, Irf4 and Gfi1 were slightly higher in Vc1+cd T cells than Vc4+cd T cells after PMA/Ionomycin treatment. Taken together, these findings indicate that both Vc1+ and Vc4+cd T cells maintain phenotypes producing IFN-γ, TNFα, TGF-β and IL-10. However, Vc1+cd T cells tend to produce Th2 type cytokine while Vc4+cd T cells preferentially produce IL-17 (Figure 6).

Discussion

Phylogenetic analysis suggests γδ T cells are precursors to modern B and αβ T cells [22]. γδ T cells are divided into subsets based on composition of T cell receptors. Interestingly, γδ T cell subsets demonstrate bias in carrying out particular functions [1]. Previously, Jutila et al. analyzed gene expression profiles of bovine CD8+ and CD8– γδ T cells using microarray and serial analysis of gene expression (SAGE) technology. They concluded inherent gene expression differences in subsets defined their distinct functional responses [23,24]. In addition, Kress et al. found considerable inherent differences in gene expression among subsets of post PMA/Ionomycin or LPS treatment of circulating Vδ1 and Vδ2 subsets in humans [18]. Vγ1+ and Vγ4+ γδ T cells are major subpopulations of peripheral γδ T cells in mice. Although global gene expression profiles of all emergent γδ thymocyte subsets have been reported by the Immunological Genome (ImmGen) Project and much knowledge has been obtained about the early divergence of gene expression programs between different γδ thymocyte subsets [12], a comprehensive gene expression profiles analysis of peripheral Vγ1+ and Vγ4+ γδ T cells isn’t available. A major hurdle has been

### Table 5. Significantly changed genes between resting and activated Vγ1+ γδ T cells enriched for KEGG pathways.

| Term                                               | Count | P-Value         |
|----------------------------------------------------|-------|-----------------|
| Cytokine-cytokine receptor interaction             | 45    | 1.50E-05        |
| Jak-STAT signaling pathway                         | 29    | 3.80E-04        |
| Hematopoietic cell lineage                         | 19    | 6.80E-04        |
| Glutathione metabolism                             | 14    | 8.20E-04        |
| Apoptosis                                           | 19    | 1.10E-03        |
| Intestinal immune network for IgA production       | 14    | 1.20E-03        |
| Prostate cancer                                    | 19    | 1.60E-03        |
| Small cell lung cancer                              | 18    | 2.10E-03        |
| Pathways in cancer                                  | 46    | 4.40E-03        |
| p53 signaling pathway                              | 15    | 4.40E-03        |
| Bladder cancer                                      | 11    | 4.70E-03        |
| Glycosphingolipid biosynthesis                      | 8     | 5.40E-03        |
| Pyrimidine metabolism                              | 18    | 7.80E-03        |
| Endometrial cancer                                  | 12    | 8.10E-03        |
| Arrhythmogenic right ventricular cardiomyopathy    | 15    | 9.50E-03        |
| Natural killer cell mediated cytotoxicity           | 21    | 9.80E-03        |
| One carbon pool by folate                          | 6     | 1.30E-02        |
| Type 1 diabetes mellitus                           | 13    | 1.30E-02        |
| Colorectal cancer                                   | 16    | 1.40E-02        |
| Melanoma                                            | 14    | 1.40E-02        |
| Glioma                                              | 13    | 1.50E-02        |
| Allograft rejection                                 | 12    | 1.80E-02        |
| Chemokine signaling pathway                         | 27    | 2.00E-02        |
| Phosphatidylinositol signaling system               | 14    | 2.20E-02        |
| ABC transporters                                    | 10    | 2.30E-02        |
| Non-small cell lung cancer                          | 11    | 2.80E-02        |
| Asthma                                              | 8     | 3.10E-02        |
| Insulin signaling pathway                           | 21    | 3.40E-02        |
| Biosynthesis of unsaturated fatty acids             | 7     | 3.70E-02        |
| Fc gamma R-mediated phagocytosis                    | 16    | 4.10E-02        |
| Graft-versus-host disease                           | 11    | 4.30E-02        |
| ECM-receptor interaction                            | 14    | 4.60E-02        |

Database for Annotation, Visualization and Integrated Discovery (DAVID), was used to analyze biological pathways associated with the differentially expressed gene transcripts. 1,995 transcripts that were identified to be related to the activation of Vγ1+ γδ T cells were enriched for 32 KEGG pathways (p < 0.05). KEGG, Kyoto Encyclopedia of Genes and Genomes. doi:10.1371/journal.pone.0112964.t005
the limited number of cells that can be obtained from healthy mice.

In this study, we resolved the limited cell count issue by establishing a primary culture method expanding Vγ1+ and Vγ4+ γδ T cells simultaneously from a single pool of mouse splenocytes. Our results proved that in vitro TCR-induced expansion for a week did not significantly change the proportion of Vγ1+ and Vγ4+ γδ T cells. We provide a comprehensive gene expression profile of mouse peripheral Vγ1+ and Vγ4+ γδ T cells in the resting and activated state. Although Vγ1+ and Vγ4+ γδ T cells share similar transcript profiles, we identified subset specific genes defining characteristics of each subset.

We identified 24 transcripts differentially expressed in resting Vγ1+ and Vγ4+ γδ T cells, and 20 transcripts differentially expressed after PMA/Ionomycin treatment. Consistent with γδ thymocytes, expression levels of Rorc, Sox13 and Scart2 were higher in Vγ4+ γδ T cells compared with Vγ1+ γδ T cells [12]. Rorc expression is reported in γδ T cells, Th22 cells, NKT cells, CD4+ CD8+ thymocytes, and others that do not belong to the T or B cell lineage [25–26]. Rorc is recognized as a lineage-specific transcription factor of Th17 and is also required for IL-17 production [29]. Transcription factor Sox13 serves a general role in the differentiation of γδ T cells [30]. Moreover, Gray et al. reported that Sox13 was indispensable for the maturation of Vγ4+ Th17 cells [31,32]. Scavenger receptor Scart2 is a marker of γδ T cells prepared to secrete IL-17A [12,31,33,34]. Our data showing Vγ4+ γδ T cells compared with Vγ1+ γδ T cells produce significantly more IL-17A and IL-17F after PMA/Ionomycin treatment are also consistent with findings in γδ thymocytes [12]. Our findings show Vγ1+ γδ T cells produce significantly more IL-4 and IL-5 after PMA/Ionomycin treatment compared with Vγ4+ γδ T cells. This finding is consistent with earlier reports showing Vγ1+ γδ T cells preferentially produce IL-4, and the depletion of Vγ1+ subset cells increases host resistance against Listeria monocytogenes infection [33]. It is important to note that Vγ1+ γδ T cells suppress Vγ4+ γδ T cell mediated antitumor function through IL-4 [36].

Alternative splicing plays an important role in increasing functional diversity of eukaryotes. Compared with the ImmGen Project, one of the advantages of RNA-seq is able to quantify

### Table 6. Significantly changed genes between resting and activated Vγ4+ γδ T cells enriched for KEGG pathways.

| Term                           | Count | P-Value  |
|-------------------------------|-------|----------|
| Cytokine-cytokine receptor interaction | 50    | 1.00E-06 |
| Biosynthesis of unsaturated fatty acids | 10    | 8.50E-04 |
| Hematopoietic cell lineage     | 19    | 1.30E-03 |
| Intestinal immune network for IgA production | 14    | 2.00E-03 |
| Jak-STAT signaling pathway     | 28    | 2.00E-03 |
| Pathways in cancer             | 49    | 2.90E-03 |
| Prostate cancer                | 19    | 3.00E-03 |
| Small cell lung cancer         | 18    | 3.90E-03 |
| Colorectal cancer              | 18    | 4.40E-03 |
| Arrhythmogenic right ventricular cardiomyopathy | 16    | 6.30E-03 |
| Glycosphingolipid biosynthesis | 8     | 7.30E-03 |
| p53 signaling pathway          | 15    | 7.40E-03 |
| Dilated cardiomyopathy         | 18    | 8.80E-03 |
| Apoptosis                      | 17    | 1.10E-02 |
| Endometrial cancer             | 12    | 1.20E-02 |
| Chemokine signaling pathway    | 29    | 1.30E-02 |
| Non-small cell lung cancer     | 12    | 1.60E-02 |
| One carbon pool by folate      | 6     | 1.70E-02 |
| Melanoma                       | 14    | 2.20E-02 |
| Glioma                         | 13    | 2.30E-02 |
| Amyotrophic lateral sclerosis (ALS) | 12    | 2.40E-02 |
| Pyrimidine metabolism          | 17    | 2.80E-02 |
| Glutathione metabolism         | 11    | 3.10E-02 |
| Toll-like receptor signaling pathway | 17    | 3.60E-02 |
| Chronic myeloid leukemia       | 14    | 3.70E-02 |
| Purine metabolism              | 24    | 3.90E-02 |
| Regulation of actin cytoskeleton | 31   | 4.10E-02 |
| Endocytosis                    | 29    | 4.50E-02 |
| Type I diabetes mellitus       | 12    | 4.60E-02 |

Database for Annotation, Visualization and Integrated Discovery (DAVID), was used to analyze biological pathways associated with the differentially expressed gene transcripts. 2,158 transcripts that were identified to be related to the activation of Vγ4+ γδ T cells were enriched for 29 KEGG pathways (p<0.05). KEGG, Kyoto Encyclopedia of Genes and Genomes.

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Table 7. Expression levels for specific genes identified by RNA-seq in both resting Vγ1+ and Vγ4+ γδ T cells.

| Category                        | Expression levels |
|---------------------------------|-------------------|
|                                 | +++++ | +++ | ++ | +   |
| Cytokine/chemokine/similar      |        |     |    |     |
| CCL4                            | ++    | CCL1 | ++  | CCR1 |
| CCL5                            | ++    | CCL3 | ++  | CCR10|
| CCR2                            | +     | CCR7 | ++  | CCR4 |
| CCR5                            | +     | CCR2 | ++  | CCR8 |
| CXCR3                           | +     | CXCR4| ++  | CCR1 |
| CXCR6                           | +     | CCL5 | +   | CCR2 |
| IL2rb                           | +     | IL12RA| +  | CCR1 |
| IL7R                            | +     | IL16 | ++  | CCR4 |
| IFNγR1                          | +     | IFNγ | ++  | CCR1 |
| IL2Rg                           | +     | IL18 | ++  | CXCR3|
| TGFB1                           | +     | TGFB1| ++  | CXCR6|
| CD2                             |       | CD164| CD1d1| CD1d2|
| CD27                            |       | CD247| CD226| CD220|
| CD97                            |       | CD96 | CD244| CD320|
| CD93                            |       | CTLA4| CD274| CD38 |
| NK cell related                 |       |       |     |      |
| KLR1; NKG2A                      |       | KLR1; NKG2D| KLR2; NKG2C |
| KLRD1; CD94                     |       | CD160; BY55| KLR3; NKG2E |
| Integlin                        |       | ITGB7; Ly69| ITGA4; Cd49D |
| LGY7; Ly69                      |       | ITGA4; Cd49D| ITGAX; Cd11c |
| ITGAV; Cd51                     |       | ITGAX; Cd11c| ITGAD; Cd11d |
| CD29                            |       | ITGAV; Cd51| ITGAD; Cd11d |
| Miscellaneous                   |       |       |     |      |
| GZMA                            |       | GZMC | FASL | TLR1 |
individual transcript isoforms and identify differentially expressed transcripts between Vγ1⁺ and Vγ4⁺ γδ T cells. We found Bclaf1 and Atf2 were preferentially expressed in Vγ4⁺ γδ T cells while Hmga1 and Bcl11b were preferentially expressed in Vγ1⁺ γδ T cells. As a transcriptional repressor, Bclaf1 interacts with several members of the Bel2 protein family and plays a role in the regulation of apoptosis and DNA repair [37,38]. Bclaf1 also plays an important role in lymphocyte homeostasis and activation [39].

Atf2 transcription factor is a member of the leucine zipper family of DNA binding proteins and forms a homodimer or a homogeneous tetramer.

### Table 7. Cont.

| Expression levels | ++++ | +++ | ++ | + |
|------------------|------|-----|----|--|
| Gzmb             |      |     |    |  |
| Gzmk             |      |     |    |  |
| Tlr12            |      |     |    |  |
| Tnf6             |      |     |    |  |

According to the expression abundance, transcripts with RPKM value over 1 were divided into 4 categories: “+” (1–10 RPKM), “++” (10–50 RPKM), “+++” (50–100 RPKM), and “++++” (>100 RPKM). RPKM, Reads Per Kilo bases per Million reads.

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### Table 8. Expression levels of significantly changed genes identified by RNA-seq in both Vγ1⁺ and Vγ4⁺ γδ T cells after PMA/Inomycin treatment.

| Expression levels | ++++ | +++ | ++ | + | - |
|------------------|------|-----|----|--|--|
| Cytokine/chemokine/similar |      |     |    |  |   |
| XCL1             |      |     |    |  |   |
| CCL9             |      |     |    |  |   |
| CXCR3            |      |     |    |  |   |
| CXCL14           |      |     |    |  |   |
| CXCL4           |      |     |    |  |   |
| CCL3             |      |     |    |  |   |
| Tnfsf11          |      |     |    |  |   |
| CCL4             |      |     |    |  |   |
| Tnfsf12a         |      |     |    |  |   |
| Tnfrsf11         |      |     |    |  |   |
| Il10             |      |     |    |  |   |
| Tnfrsf9          |      |     |    |  |   |
| Tnf6             |      |     |    |  |   |

According to the expression abundance, transcripts were divided into 5 categories: “-” (<1 RPKM), “+” (1–10 RPKM), “++” (10–50 RPKM), “+++” (50–100 RPKM), and “++++” (>100 RPKM). RPKM, Reads Per Kilo bases per Million reads.

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heterodimer with c-Jun, stimulating cAMP responsive element (CRE) dependent transcription. Aft2 expression is lower in CD8+ T cells compared with CD4+ T cells, a functional explanation to the differential response to glucocorticoids between CD8+ and CD4+ T cells [40]. As an architectural chromatin factor, Hmgal binds preferentially to the minor groove of AT rich regions in double stranded DNA. It is involved in many cellular processes including regulation of inducible gene transcription, insulin resistance, diabetes and malignant transformation [41,42]. Nakao et al. revealed a new role for Hmgal1 in transcriptional silencing in T cell lineages and leukemic cells [43]. However, the roles of Bcl11b, Aft2 and Hmgal1 in γδ T cells have not been reported. Bcl11b is a T-cell specific gene and required for T-lineage commitment. Afferrent expression of Bcl11b contributes to human T-ALL [44]. In contrast with ImmGen Project results showing Bcl11b was preferentially expressed in Vγ4+ γδ thymocytes [12], we identified one transcript isoform of Bcl11b preferentially expressed in activated Vγ1+ γδ T cells. The role of the transcript isoform of Bcl11b in Vγ1+ γδ T cells needs further study.

Many of the differentially expressed gene transcripts identified in activated Vγ1+ and Vγ4+ γδ T cells shared similar signaling pathways. We found higher expression of IL-4 and IL-5 in activated Vγ1+ γδ T cells. This suggests a role in asthma given that Vγ4+ γδ T cells suppress airway hyperresponsiveness, compared with Vγ1+ γδ T cells that enhance airway hyperresponsiveness and raise levels of Th2 cytokines and eosinophils infiltration in the airways [8,9,45].

Both resting Vγ1+ and Vγ4+ γδ T cells exhibited high levels of transcripts for several chemokines and chemokine receptors, including CCL4, CCL5, CCR2, CCR5 and CXCR3. These data highlight the role of Vγ1+ and Vγ4+ γδ T cells in immune-regulatory and inflammatory processes. For example, CCL4 (MIP-1beta) and CCL5 (RANTES) are both Th1-associated chemokines that bind to CCR5. Up-regulation of CCR5 ligands may play a role in the recruitment process of blood monocytes, memory T helper cells and eosinophils. CCR2 is expressed on both Vγ1+ and Vγ4+ γδ T cells, and is necessary for the accumulation of γδ TILs to the tumor bed [46]. It is interesting to note that CXCR6 was previously thought to be expressed in human Vδ2 cells, but not Vδ1 cells [18]. However, we found high CXCR6 levels in both Vγ1+ and Vγ4+ γδ T cells. CXCR6 plays a critical role in NK cell memory of haptns and viruses [47]. Whether CXCR6 plays a role in Vγ1+ and Vγ4+ γδ T memory cells needs further examination.

Integrins play key roles in immune responses, leukocyte trafficking and many human diseases. Most integrin related research has been focused on γδ T cells, with little published on γδ T cells. Our results show several integrins were highly expressed in Vγ1+ and Vγ4+ γδ T cells. For example, β5a (Cd103), implicated in epithelial T cell retention, is highly expressed on Vγ1+ and Vγ4+ γδ T cells [48]. β5a contributes to clustering and activation of Vγ TCRs expressed by epidermal T cells [49]. Signals mediated by integrins play important roles in the activation of T cells [50]. Therefore, we suggest stimulating integrin expression provides a costimulation signal, increasing the sensitivity of γδ T cell activation.

PMA/Ionomycin induces a robust non-TCR mediated response in Vγ1+ and Vγ4+ γδ T cells. We show after PMA/Ionomycin treatment several activation markers of T cells were upregulated including CD25, CD69 and CD44, along with most cytokine genes in both subsets. In addition, activated Vγ1+ and Vγ4+ γδ T cells produced high levels of XCL1, CCL3, CCL4, CCL1, IFN-γ, TNFα, Lta, Cd2 and IL-10. IFN-γ and TNFα are Th1 type cytokines. Previous reports show Vγ1+ γδ T cells are the major γδ T subset producing IFN-γ, and they steer CD4+ T cells toward a dominant Th1 cell response [7,51,52]. Moreover, He et al. reported that CD44 rich Vγ4+ γδ T cells produced significantly more IFN-γ compared with Vγ1+ γδ T cells, partly due to the high expression level of eomesodermin [16]. In contrast, Matsuzaki et al. reported that Vγ1+ γδ T cells were the major γδ T subset producing IFN-γ in response to L. monocytogenes infection [53]. The opposing results are likely due to different disease models and treatment methods. A separate study reported higher levels of IL-10 in human Vδ1 cells compared with Vδ2 cells [18]. However, our results show both Vγ1+ and Vγ4+ γδ T cells produce high levels of IL-10.

Narayan et al. reported that Vγ4+ γδ thymocytes expressed high levels of Stat4, Maf, Gat4 and Eomes compared with Vγ1+ γδ thymocytes [12]. However, our results show both Vγ1+ and Vγ4+ γδ T cells expressed high levels of these transcription factors and the levels of Gat4 were slightly higher in Vγ1+ γδ T cells compared with Vγ4+ γδ T cells after PMA/Ionomycin treatment. Gat4 is critical for Th2 cell differentiation and required for IL-4 production. The higher level of Gat4 expression in Vγ1+ γδ T
cells is consistent with the phenotype of Vγ1* Vδ T cells producing more IL-4 than Vγ4* Vδ T cells. T-bet is a major factor for Th1 cell differentiation and IFN-γ production [54]. Eomes is also involved in Th1 differentiation and IFN-γ production [53]. The upregulation of Tbet and Eomes is consistent with the phenotype of both Vγ1* Vδ T and Vγ4* Vδ T cells that produce high levels of IFN-γ. The difference between our results with the ImmGen Project may be due to the source of Vδ T cells. The cells used in the ImmGen Project are γδ thymocytes, however the cells in our study were peripheral γδ T cells derived from the spleen.

Taken together, this study shows both Vγ1* and Vγ4* γδ T cells maintain inflammatory and regulatory phenotypes. Both demonstrate an inflammatory cell phenotype via IFN-γ and TNFα expression. And, both display a regulatory cell phenotype via IL-10 production. Vγ1* γδ T cells produced more Th2 type cytokines, while Vγ4* γδ T cells tended to produce more IL-17. Thus, Th2 type cytokines may explain how Vγ1* γδ T cells affect anti-inflammatory functions in different infection models, and describe the enhancing effect on airway hyperresponsiveness (AIR) [56]. IL-17 cytokines support the pro-inflammatory function of Vγ4* γδ T cells in the infection models and the inhibitory effect on airway hyperresponsiveness (AIRh). Although this study was performed in Vγ1* and Vγ4* γδ T cells expanded in vitro, which may not fully represent the true status of Vγ1* and Vγ4* γδ T cells in vivo, our results support the hypothesis that distinct γδ TCR types direct cells to acquire a certain type of functional programming during thymic development [57].

Complementary to the ImmGen Project, this report provides a comprehensive gene expression profile of mouse peripheral Vγ1* and Vγ4* γδ T cells following PMA/Ionomycin treatment. Although both γδ T cell populations have similar transcript profiles, subset-specific transcripts define distinct characteristics and describe the inherent differences between Vγ1* and Vγ4* γδ T cells.

Supporting Information
Dataset S1 Raw data and differential expression analysis in RNA-seq. (XLSX)
Dataset S2 Differentially expressed genes between the resting and activated Vγ1* γδ T cells. (XLSX)
Dataset S3 Differentially expressed genes between the resting and activated Vγ4* γδ T cells. (XLSX)
Dataset S4 Transcription factors related to Th cell differentiation and cytokine secretion. (XLSX)

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Author Contributions
Conceived and designed the experiments: WH SZ JZ PD. Performed the experiments: PD SZ MC. Analyzed the data: WH PD LC. Contributed reagents/materials/analysis tools: PD SZ NK YH. Contributed to the writing of the manuscript: WH JZ PD.

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