β-Cell–Specific CD8 T Cell Phenotype in Type 1 Diabetes Reflects Chronic Autoantigen Exposure

Ania Skowera,1 Kristin Ladell,2 James E. McLaren,2 Garry Dolton,2 Katherine K. Matthews,2,3 Emma Gostick,2 Deborah Kronenberg-Versteeg,1 Martin Eichmann,1 Robin R. Knight,1 Susanne Heck,4 Jake Powrie,5 Polly J. Bingley,6 Colin M. Dayan,7 John J. Miles,2,3,8 Andrew K. Sewell,2 David A. Price,2 Emma Gostick,2 Deborah Kronenberg-Versteeg,1 Martin Eichmann,1 Robin R. Knight,1 Susanne Heck,4 Jake Powrie,5 Polly J. Bingley,6 Colin M. Dayan,7 John J. Miles,2,3,8 Andrew K. Sewell,2 David A. Price,2

Autoreactive CD8 T cells play a central role in the destruction of pancreatic islet β-cells that leads to type 1 diabetes, yet the key features of this immune-mediated process remain poorly defined. In this study, we combined high-definition polychromic flow cytometry with ultrasensitive peptide–human leukocyte antigen class I tetramer staining to quantify and characterize β-cell–specific CD8 T cell populations in patients with recent-onset type 1 diabetes and healthy control subjects. Remarkably, we found that β-cell–specific CD8 T cell frequencies in peripheral blood were similar between subject groups. In contrast to healthy control subjects, however, patients with newly diagnosed type 1 diabetes displayed hallmarks of antigen-driven expansion uniquely within the β-cell–specific CD8 T cell compartment. Molecular analysis of selected β-cell–specific CD8 T cell populations further revealed highly skewed oligoclonal T cell receptor repertoires comprising exclusively private clonotypes. Collectively, these data identify novel and distinctive features of disease-relevant CD8 T cells that inform the immunopathogenesis of type 1 diabetes.

Type 1 diabetes is an autoimmune disease characterized by T cell–mediated destruction of insulin-producing β-cells in the islets of Langerhans (1,2). Several lines of evidence implicate the CD8 T cell lineage in this process: 1) CD8 T cells predominate in islet-centered leukocytic infiltrates close to diagnosis (3,4); 2) autoreactive CD8 T cells with β-cell epitope specificities have been detected in such early infiltrates (3); 3) CD8 T cell clones specific for preproinsulin-derived peptides can kill β-cells in vitro (5,6); and 4) large genetic association studies link disease susceptibility to the inheritance of specific human leukocyte antigen class I (HLA) alleles (7). This mounting functional and epidemiological evidence, combined with the expanding array of reported HLAI-restricted β-cell epitopes (8,9), provides a strong rationale for detailed studies of autoreactive CD8 T cells in type 1 diabetes.

Technological advances have facilitated the design of CD8-centric studies, enabling enhanced data retrieval from cell-limited samples to illuminate fundamental immunobiological processes. In particular, antigen-specific CD8 T cells can now be enumerated routinely by flow cytometry irrespective of functional outputs due to the advent of recombinant peptide–HLAI (pHLAI) proteins in various fluorochrome-tagged multimeric formats (10–13). Moreover, developments in instrumentation and fluorochrome technology continue to expand the horizons of

1Department of Immunobiology, King’s College London School of Medicine, London, U.K.
2Institute of Infection & Immunity, Cardiff University School of Medicine, Cardiff, U.K.
3QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia
4National Institute for Health Research Biomedical Research Centre at Guy’s and St Thomas’ National Health Service Foundation Trust and King’s College London, London, U.K.
5Department of Diabetes and Endocrinology, Guy’s and St Thomas’ National Health Service Foundation Trust and King’s College London, London, U.K.
6School of Clinical Sciences, University of Bristol, Bristol, U.K.
7School of Molecular & Experimental Medicine, Cardiff University School of Medicine, Cardiff, U.K.
8School of Medicine, The University of Queensland, Brisbane, Queensland, Australia

Corresponding author: Ania Skowera, a.skowera@qmul.ac.uk.
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A.S., K.L., D.A.P., and M.P. contributed equally to this study.
A.S. is currently affiliated with the Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, U.K.
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polychromatic flow cytometry (14,15), facilitating the identification of functionally distinct T cell subsets across a spectrum of phenotypic heterogeneity (16). The collective application of such innovations has transformed our understanding of T cell ontogeny in response to infectious “foreign” antigens. However, it is unclear whether the emerging conceptual frameworks extend similarly to autoimmune processes.

Although β-cell epitope-specific CD8 T cell expansions have been identified in the peripheral blood of patients with type 1 diabetes (6,17), the functional and phenotypic properties of these cells in the context of disease relevance remain largely uncharacterized. This is a significant knowledge gap for two important reasons. First, it cannot be assumed that autoreactive CD8 T cells will follow the rules of antigen engagement established in previous studies. Indeed, autoreactive T cell receptors (TCRs) characteristically display low-affinity interactions with their cognate pHLA antigens (18,19), presumably reflecting the effects of thymic culling to eliminate potentially dangerous self-specific clonotypes from the peripheral repertoire. Moreover, autoantigens are expressed continuously and guarded by peripheral tolerance mechanisms designed to limit cognate T cell expansion (1). Second, immune intervention strategies designed specifically to target either effector T cells or innate inflammatory pathways that could impact adaptive immune responses are currently being trialed in type 1 diabetes (20–23). The identification of T cell–related biomarkers could facilitate immune monitoring in this setting and delineate correlates of therapeutic efficacy. Accordingly, we undertook a multiparametric analysis of β-cell–specific CD8 T cell populations in patients with type 1 diabetes and healthy control subjects to identify the key cellular features associated with disease.

**RESEARCH DESIGN AND METHODS**

**Study Subjects**
The study cohort comprised 14 HLA-A*0201+ patients (mean age, 30 years ± SD 6.4) with newly diagnosed type 1 diabetes (mean disease duration, 4 months) and 14 HLA-A*0201+ healthy control subjects (mean age, 30 years ± SD 5.0). Autoantibodies against GAD65 and IA-2 were detected in 64 (9 of 14) and 71% (10 of 14) of patients in the type 1 diabetic group, respectively. Local research ethics committee approval (National Research Ethics Committee, Bromley NRES Committee, reference number 08/H0805/14) was granted at each participating center, and written informed consent was obtained in all cases.

**Blood Samples**
Fresh venous blood was collected into heparinized tubes and transported for processing within 3 h of collection. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation (Nycomed), washed twice in RPMI 1640 supplemented with 1% penicillin/streptomycin and 2% human AB serum (all Life Technologies), and then resuspended in freezing medium comprising 90% heat-inactivated, filter-sterilized FBS and 10% DMSO (Sigma-Aldrich). Aliquots of 10–20 × 10^6 cells/mL per vial were cooled overnight at a controlled rate of −1°C/min to −80°C prior to storage in liquid nitrogen. All samples were analyzed within 2 years of cryopreservation.

**Tetrameric pHLAI Complexes**
Soluble, fluorochrome-conjugated pHLA-A*0201 tetramers were produced as described previously (24). Incorporated peptides were synthesized at >95% purity (BioSynthesis). The corresponding epitopes are summarized in Table 1.

**Polychromatic Flow Cytometry**
Thawed PBMCs were pretreated with 50 nmol/L dasatinib before tetramer staining to enhance the detection of low-avidity T cells as described previously (25). The following monoclonal antibodies were used for phenotypic analysis: 1) anti-CD3–H7APC, anti-CD4–V500, anti-CD45RO–PECy7, anti-CD57–FITC, anti-CD95–PE, and anti-CCR7–PerCP/Cy5.5 (BD Biosciences); 2) anti-CD8–QD705 (Life Technologies); and 3) anti-CD27–PECy5 (Beckman Coulter). Dead cells were excluded from the analysis using the amine-reactive dye ViViD (Life Technologies); monocytes and B cells were eliminated in the same dump channel after staining with anti-CD14–Pacific Blue and anti-CD19–Pacific Blue, respectively (Life Technologies). Stained

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<td>Insulin B chain</td>
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<td>Islet-specific glucose-6-phosphatase catalytic subunit-related protein</td>
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<td>CMV pp65</td>
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<td>EBV BMLF1</td>
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*Numbers of healthy control subjects/ type 1 diabetic patients studied for each epitope specificity.
samples were acquired using an LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo version 9.4 (Tree Star Inc.). The gating strategy is illustrated in Supplementary Fig. 1. For quality-control purposes, tetramers were batch-tested prior to experimentation using specific CD8 T cell clones where available (5). Assay variability was monitored throughout the study using aliquots of cryopreserved PBMCs drawn from a single healthy donor at a single time point (Supplementary Fig. 2).

TCR Clonotyping

Clonotypic analysis of antigen-specific CD8 T cell populations was performed as described previously with minor modifications (26). Briefly, 222–1,071 viable tetramer-labeled CD3⁺CD8⁺ T cells were sorted directly ex vivo into 1.5-mL microtubes (Sarstedt) containing 100 μL RNAlater (Applied Biosystems) using a custom-modified FACSAria II flow cytometer (BD Biosciences). Unbiased amplification of all expressed TRB gene products was conducted using a template-switch anchored RT-PCR with a 3′ constant region primer (5′-TGGCTCAA CAAGGAGACCT-3′). Amplicons were subcloned, sampled, sequenced, and analyzed as described previously (24). The ImMunoGeneTics database nomenclature is used in this report (27).

Statistical Analysis

Single experimental variables were analyzed using the Mann-Whitney U test or the Wilcoxon signed-rank test in GraphPad Prism 5 (GraphPad Software). Multivariate analyses of flow cytometric data were performed using the probability binning algorithm in FlowJo version 9.7.2 (Tree Star Inc.).

RESULTS

Ex Vivo Identification of β-Cell-Specific CD8 T Cells

To ensure the optimal detection of autoreactive β-cell-specific CD8 T cell populations directly ex vivo, we conducted extensive pilot experiments with dasatinib, a reversible protein kinase inhibitor that lowers the TCR affinity threshold required for tetramer binding at the cell surface (25). This approach enables the visualization of low-avidity CD8 T cells that would otherwise remain undetectable and enhances the intensity of tetramer staining via active inhibition of TCR downregulation. In line with our previous findings, we observed greater frequencies of β-cell-specific CD8 T cells in all test subjects after sample pretreatment with dasatinib (Fig. 1A–E) and clearer separation of tetramer-labeled events across all specificities (Fig. 1A–G).

Next, we used this approach to compare β-cell-specific CD8 T cell frequencies in newly diagnosed type 1 diabetic patients and healthy nondiabetic control subjects across

![Figure 1](image-url)

Figure 1—Identification of antigen-specific CD8 T cell populations. Thawed PBMCs were stained with pHLA-A*0201 tetramers representing PPI15–24 (A), InsB10–18 (B), IGRP265–273 (C), IA-2797–805 (D), GAD65114–123 (E), CMV pp65495–503 (F), and EBV BMLF1280–288 (G). Gates were set serially on singlets, live CD3⁺CD14⁻CD19⁻ cells, and lymphocytes prior to Boolean exclusion of dye aggregates and subsequent analysis in bivariate CD8 versus tetramer plots (Supplementary Fig. 1A). Representative paired data in the absence or presence of dasatinib are shown for type 1 diabetic patients and healthy nondiabetic control subjects. Tetramer-binding CD8 T cell frequencies are indicated. PKI, protein kinase inhibitor.
five distinct HLA-A*0201–restricted specificities (PPI15–24, InsB10–18, IGRP265–273, IA-2797–805, and GAD65114–123). Two pHLA-A*0201 tetramers representing immunodominant epitopes from common persistent herpesviruses (cytomegalovirus [CMV] pp65495–503 and Epstein-Barr virus [EBV] BMLF1280–288) were also included for control purposes (Table 1). Again, increased β-cell–specific CD8 T cell frequencies were observed for the majority of subjects in the presence of dasatinib, reaching statistical significance for most specificities in each subject group (Fig. 2). Although less striking, we noted a similar frequency enhancement for CMV pp65495–503 tetramer-binding CD8 T cells. However, this most likely reflects improved visualization of nonamplified precursors close to the technical limit of detection rather than a biologically relevant phenomenon within the antigen-experienced pool. Consistent with this interpretation, no such differences were observed for EBV BMLF1280–288 tetramer-binding CD8 T cells, which are driven to expand in the majority of HLA-A*0201+ donors as a consequence of high viral prevalence. More importantly, there were no significant differences between type 1 diabetic patients and healthy control subjects with respect to CD8 T cell frequencies across any of the seven incorporated specificities, either in the presence or absence of dasatinib. Accordingly, we hypothesized that disease-related differences in autoreactive CD8 T cell populations may reflect an antigen-driven inflammatory process that does not manifest in simple numerical terms, at least within the peripheral circulation.

**β-Cell–Specific CD8 T Cells Are More Differentiated in Type 1 Diabetic Patients**

To examine the phenotypic characteristics of β-cell–specific CD8 T cells, we constructed a polychromatic flow

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Figure 2—Summary of antigen-specific CD8 T cell frequencies. Thawed PBMCs were stained with pHLA-A*0201 tetramers representing PPI15–24 (A), InsB10–18 (B), IGRP265-273 (C), IA-2797–805 (D), GAD65114–123 (E), CMV pp65495–503 (F), and EBV BMLF1280–288 (G). Graphs show tetramer-binding CD8 T cell frequencies in the absence (empty symbols) or presence (filled symbols) of dasatinib for type 1 diabetic patients (T1D) and healthy control subjects (CTR). No significant differences across identical comparisons were observed between subject groups. Bars represent median values. Statistical analyses were performed using the Wilcoxon signed-rank test; *P* values ≤0.05 are shown.
cytometry panel designed to exclude irrelevant events (ViViD, CD14, and CD19), assign lineage (CD3, CD4, and CD8), and define differentiation status (CD27, CD45RO, CD57, CD95, and CCR7). Across all pooled β-cell specificities, we found that the percentage of autoreactive CD8 T cells with a naive phenotype ($T_N$: CD27$^+$CD45RO$^-$CD57$^-$CD95$^-$CCR7$^+$) was significantly lower in type 1 diabetic patients compared with healthy control subjects ($P < 0.0001$; Fig. 3). This pattern held within individual specificities, reaching significance for PPI$_{15-24}$ ($P = 0.02$), IGRP$_{265-273}$ ($P = 0.01$), and IA-2$_{797-805}$ ($P = 0.02$). Importantly, no such differences were detected between groups for either of the virus-derived specificities. Moreover, total naïve CD8 T cell frequencies were similar in type 1 diabetic patients and healthy control subjects (Supplementary Fig. 3). Of note, a large proportion of CD8 T cells specific for the CMV pp65$_{95-503}$ epitope displayed a classical naive phenotype. In contrast, very few CD8 T cells specific for the EBV BMLF1$_{280-288}$ epitope were naïve. These observations substantiate the interpretation above that dasatinib-mediated frequency amplification within the CMV specificity reflects enhanced precursor detection in seronegative individuals.

Collectively, these data suggest that recent-onset type 1 diabetes is characterized by antigen-driven expansion of β-cell–specific CD8 T cells into more differentiated compartments, likely facilitated by tissue-specific inflammatory processes. Consistent with this notion, greater proportions of CD8 T cells with a stem cell memory phenotype ($T_{SCM}$: CD27$^+$CD45RO$^-$CD95$^+$CCR7$^+$) were present in type 1 diabetic patients compared with healthy control subjects across all pooled β-cell specificities ($P = 0.025$), as well as individually within the autoreactive populations specific for PPI$_{15-24}$ ($P = 0.05$) and InsB$_{10-18}$ ($P = 0.029$). Moreover, single-marker analyses revealed that β-cell–specific CD8 T cells expressed higher frequencies of CD57 ($P = 0.0002$) and CD95 ($P < 0.0001$) in type 1 diabetic patients compared with healthy control subjects (Fig. 4). These surface proteins demarcate terminal differentiation and memory status, respectively (16,29).

Conversely, β-cell–specific CD8 T cells less frequently expressed CD27 and CCR7 in type 1 diabetic patients compared with healthy control subjects ($P = 0.0002$ and $P = 0.005$, respectively). These markers classically delineate naïve and early memory T cells (30,31). No single-marker differences between subject groups were observed for either of the virus-derived specificities or CD8 T cells as a whole.

To extend these findings, we used frequency difference gating and probability binning to conduct multivariate

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**Figure 3**—Phenotypic subset analysis of antigen-specific CD8 T cells. A: Pie chart representations of mean subset percentages for pooled β-cell–specific CD8 T cells and individual specificities in type 1 diabetic patients (T1D; top row) and healthy control subjects (CTR). Subsets are defined and color coded as follows: green, $T_N$ (CD27$^+$CD45RO$^-$CD57$^-$CD95$^-$CCR7$^+$); yellow, $T_{SCM}$ (CD27$^+$CD45RO$^-$CD95$^+$CCR7$^+$); blue, effector T cells (CD27$^-$CD45RO$^-$CD95$^+$CCR7$^+$); and red, all remaining memory cells. B: Column plots showing $T_N$ and $T_{SCM}$ subset percentages for pooled β-cell–specific CD8 T cells and individual specificities in T1D and healthy control subjects (CTR). Representative data are shown in Supplementary Fig. 1B and C. Almost identical results were obtained with the $T_N$ subset defined in the absence of CD57 (CD27$^+$CD45RO$^-$CD95$^+$CCR7$^+$). Bars represent median values. Statistical analyses were performed using the Mann–Whitney U test; $P$ values <0.05 are shown.
analyses across concatenated datasets (32,33). A phenotypically distinct CD27<sup>intermediate</sup>CD95<sup>+</sup> population of CD8 T cells was identified in type 1 diabetic patients at significantly higher frequencies compared with healthy control subjects for the PPI15<sub>–24</sub> (\(P < 0.01\)), IGRP265<sub>–273</sub> (\(P < 0.01\)), and pooled b-cell (\(P < 0.01\)) specificities (Fig. 5). The majority of these cells within the PPI<sub>15–24</sub> specificity coexpressed CD45RO and CCR7, whereas greater variability was apparent for the IGRP265–273 and pooled b-cell specificities. Expression of CD57 was heterogeneous in all cases. These data confirm the preceding observations and define a b-cell–specific early memory phenotype associated with type 1 diabetes.

**DISCUSSION**

In this study, we combined high-definition polychromatic flow cytometry with ultrasensitive pHLAI tetramer staining to define the magnitude and differentiation status of b-cell–specific CD8 T cell populations in type 1 diabetic patients and healthy control subjects. Moreover, we achieved sufficient resolution with this approach to enable direct ex vivo analysis of the autoreactive TCR repertoire in a subset of individuals. In contrast to healthy control subjects, patients with newly diagnosed type 1 diabetes displayed hallmarks of antigen-driven expansion within the b-cell–specific CD8 T cell compartment. No such differences were observed between subject groups.
for persistent viral specificities or CD8 T cells globally. Collectively, these data identify phenotypic biomarkers of disease-relevant CD8 T cell–mediated autoimmunity in type 1 diabetes.

Remarkably, we found that β-cell–specific CD8 T cell frequencies in peripheral blood were similar between type 1 diabetic patients and healthy control subjects. These findings are consistent with some previous studies of presumed autoimmune conditions (35), but discrepant with other reports in the field (6,17), most likely due to differences in detection sensitivity and cohort composition. Of particular note, the use of dasatinib to enhance tetramer-staining thresholds revealed autoreactive CD8 T cells in the current study that were not visible with standard

**Figure 5**—Frequency difference gating analysis of antigen-specific CD8 T cells. Overlays of concatenated data from type 1 diabetic patients (cloud plots) and healthy control subjects (blue dots) are shown for the indicated β-cell specificities across bivariate phenotypic profiles. Populations of CD27intermediateCD95+ CD8 T cells, present at significantly higher frequencies in type 1 diabetic patients compared with healthy control subjects, are displayed as red dots.
protocols. The reliable identification of naive precursors in addition to antigen-experienced subsets readily explains the equivalent frequencies of β-cell–specific CD8 T cells between subject groups. It is also important to recognize that peripheral CD8 T cell frequencies do not necessarily reflect the tissue-localized immune cell environment. Indeed, histopathological evaluations of β-cell–specific CD8 T cell populations in the insulitic lesions of patients who died close to the time of diagnosis have demonstrated pronounced variability in antigen targeting between islets and individuals (3,36). Accordingly, it is difficult to equate quantitative measures of immune autoreactivity in the periphery with CD8 T cell–mediated events in the pancreas.

In contrast to the lack of simple numerical correlates, we found clear phenotypic signatures of functionally relevant β-cell–derived antigen exposure in type 1 diabetic patients. Conventional subset analyses revealed fewer T_N cells and greater numbers of T_SCM cells across all pooled β-cell specificities within the CD8 compartment of patients with newly diagnosed type 1 diabetes compared with healthy control subjects. Single-marker evaluations confirmed this overall pattern, with reduced expression of CD27 and CCR7 and elevated expression of CD57 and CD95. The increased prevalence of antigen-specific T_SCM cells, which serve as a reservoir to replenish more differentiated memory subsets (16,37), could perpetuate the underlying autoimmune process of β-cell destruction. It is also notable that granzymes and perforin are strongly coexpressed with CD57, which acts accordingly as a surrogate marker of cytolytic activity (38,39). Such highly differentiated cells may therefore associate with more severe disease manifestations (40–42). Multivariate analyses further identified a phenotypically distinct CD27intermediateCD95+ population of β-cell–specific CD8 T cells in type 1 diabetic patients that was significantly less frequent in healthy control subjects, again consistent with an antigen-driven disease process as reported previously in a viral system (43). Importantly, all three analytical approaches yielded significant differences between subject groups solely within the β-cell–specific

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Individual clonotypes are represented in order of relative frequency. Gene usage and CDR3 amino acid sequence are shown in each case.
CD8 T cell compartment. Thus, the phenotypic profile of tissue-directed autoreactive CD8 T cell populations may act as a useful biomarker of disease activity in type 1 diabetes.

In further experiments, we characterized the TCR repertoire of tetramer-binding CD8 T cells specific for the PPI_{15-24} epitope. This specificity was selected on the grounds that robust phenotypic differences were detected between type 1 diabetic patients and healthy control subjects across all analytical strategies. Moreover, quantitative differences in the cognate CD8 T cell population have previously been reported to associate with disease activity (17). The enhanced detection sensitivity afforded by our approach facilitated this analysis, which represents the first ex vivo characterization of autoreactive CD8 T cell clonotypes. In all cases, we observed highly skewed oligoclonal repertoires reminiscent of virus-driven expansions (24). These hierarchical structures could reflect focused autoantigen-specific priming or cross-reactivity with an environmental trigger in the form of a highly immunogenic pathogen-derived epitope (44). However, it is important to note that this clonotypic pattern was not unique to the type 1 diabetic setting. Similar findings in two healthy control subjects, associated with a small central memory expansion in an otherwise largely naive landscape in one (control 2) and a highly differentiated predominant memory phenotype in the other (control 28), suggest the natural occurrence of heterologous responses. Thus, oligoclonal β-cell–specific CD8 T cell populations can exist in the memory pool of healthy individuals without concomitant evidence of disease activity. It is intriguing to speculate that the highly private nature of these repertoires could provide a molecular explanation for differential TCR-mediated outcomes in the immunopathogenesis of type 1 diabetes, akin to recent descriptions in viral systems (45–47).

Overall, the current study demonstrates that β-cell–specific CD8 T cells are more differentiated in patients with newly diagnosed type 1 diabetes compared with healthy control subjects. This key result identifies an immune correlate of disease activity that could be further refined at the clonotypic level to monitor organ-specific tissue damage in the periphery.

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