Epithelial cytoprotection sustains ectopic expression of tissue-restricted antigens in the thymus during murine acute GVHD

Short Title: Thymus function in acute GVHD

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Key points

1. Acute GVHD predisposes to autoimmune chronic GVHD but it is currently unclear how autoimmunity is linked to antecedent alloimmunity

2. Loss of central tolerance induction that occurs via functional compromise of thymic epithelial cells may provide such a pathogenic link

Abstract

Development of acute graft-versus-host disease (aGVHD) predisposes to chronic GVHD with autoimmune manifestations. A characteristic of experimental aGVHD is the *de novo* generation of autoreactive T cells. Central tolerance is dependent on the intrathymic expression of tissue-restricted peripheral self-antigens (TRA) which is in mature medullary TEC (mTEC\textsuperscript{high}) partly controlled by the *autoimmune regulator* (Aire). Since TECs are targets of donor T-cell alloimmunity we tested whether murine aGVHD interfered with the capacity of recipient Aire\textsuperscript{*}mTEC\textsuperscript{high} to sustain TRA diversity. We report that aGVHD weakens the platform for central tolerance induction because individual TRAs are purged from the total repertoire secondary to a decline in the Aire\textsuperscript{*}mTEC\textsuperscript{high} cell pool. Peritransplant administration of an epithelial cytoprotective agent, fibroblast growth factor-7, maintained a stable pool of Aire\textsuperscript{*}mTEC\textsuperscript{high}, with an improved TRA transcriptome despite aGVHD. Taken together our data provide a mechanism for how autoimmunity may develop in the context of antecedent alloimmunity.
Introduction

Self-tolerance of the nascent T-cell receptor repertoire is attained through negative selection in the thymus.\textsuperscript{1} Essential for clonal deletion is the exposure of thymocytes to self-antigens, including those with highly restricted tissue expression. Within the thymus the ectopic expression of many tissue-restricted peripheral self-antigens (TRA) is a distinct property of mature medullary thymic epithelial cells (mTEC\textsuperscript{high}).\textsuperscript{2,3} TRA expression is controlled partly by the transcription factor autoimmune regulator (Aire).\textsuperscript{3} Deficits in Aire and/or TRA expression (independent of their causes) impair negative selection and can consequently cause autoimmune disease.\textsuperscript{2-5}

Acute and chronic graft-versus-host disease (aGVHD and cGVHD, respectively) remain the primary complications of allogeneic hematopoietic stem cell transplantation (allo-HSCT).\textsuperscript{6} aGVHD is initiated by alloreactive donor T cells and targets a restricted set of tissues, including the thymus.\textsuperscript{7,8} The development of human aGVHD predisposes to cGVHD whose autoimmune manifestations are integral components of disease.\textsuperscript{9} It remains uncertain, however, whether and how autoimmunity is linked to antecedent alloimmunity. A hallmark of murine aGVHD is the \textit{de novo} generation of autoreactive T cells from donor HSC\textsuperscript{10,11} which can mediate the evolution from acute to chronic GVHD.\textsuperscript{12,13} Since the thymic epithelium is a target of donor T-cell alloimmunity\textsuperscript{7,8,14} we hypothesized that thymic aGVHD interferes with the mTEC\textsuperscript{high} capacity to sustain TRA diversity, thus decreasing the platform for central tolerance. In this context, interventions directed at epithelial cytoprotection are expected to maintain posttransplantation mTEC\textsuperscript{high} integrity and function. To test these two interrelated hypotheses, we used murine allo-HSCT models and investigated the effects of fibroblast growth factor-7 (Fgf7), which boosts thymopoiesis through its mitogenic action on TEC.\textsuperscript{15,16}
Methods

Female C57BL/6 (B6;H-2^b), (C57BL/6xDBA/2)F1 (BDF1;H-2^bd), Balb/c (H-2^d), CBy.PL(B6)-Thy1^a/ScrJ (Balb/c-Thy1.1;H-2^d), 129/Sv (H-2^b) and B6.SJL-PtprcaPep3b/BoyJ (B6.CD45.1;H-2^b) mice were kept in accordance with federal regulations. Thymic aGVHD was induced in unirradiated or total body irradiated (TBI) recipients14 (Figure S1). Recombinant human Fgf7 (palifermin, Kepivance®, Biovitrum, Sweden) was injected i.p. from day -3 to day +3 after allo-HSCT at a dose of 5 mg/kg/day.15,16

Confocal microscopy of thymic sections was performed using a Zeiss LSM510 (Carl-Zeiss AG, Switzerland).17 The TEC compartment was analyzed by flow cytometry (FACSAria®, Becton Dickinson, Mountain View, CA). mTEC^high were identified as cells with a CD45^EpCam^Ly51^-UEA1^-MHCII^high phenotype17-19 (Figure S2). Global gene expression profiling was used to establish TRA diversity in transplant recipients. Microarray data of triplicate samples of pure mTEC^high preparations (Mouse Gene 1.0 ST Array; Affymetrix, Santa Clara, CA; data available under accession number E-MEXP-3745 at http://www.ebi.ac.uk/arrayexpress) were verified by quantitative PCR. Gene expression data from multiple published sources20,21 and from MOE430 and GNF1M gene atlases (http://biogps.org) were combined for identification of Aire-dependent and -independent TRA (supplemental methods). Bioinformatics was implemented using software from 'The R Project for Statistical Computing' (http://www.r-project.org).

Results and discussion

Total numbers of mTEC^high declined over time in untransplanted adult BDF1 and B6 mice, confirming age-dependent changes of the thymic stroma22 (Figures 1A,B). We then investigated four mouse models of age-matched allo-HSCT. In unconditioned recipients of haploidentical HSCT (H-2^b→H-2^bd; b→bd), the induction of thymic
aGVHD\textsuperscript{14,15} (Figure S2) progressively reduced total mTEC\textsuperscript{high} numbers to ≤10\textsuperscript{3} cells/mouse in animals surviving aGVHD (Figure 1A). This observation was mirrored in allo-HSCT that included TBI before MHC-identical, -disparate or haploidentical transplantation (Figure 1B). In these models, the conditioning initially reduced mTEC\textsuperscript{high} numbers independently of aGVHD but cell loss was either more pronounced (\(b \rightarrow bd\)) or more protracted (\(d \rightarrow b; b \rightarrow b\)) in the presence of disease. Hence, reduction in mTEC\textsuperscript{high} compartment size is a universal manifestation of thymic aGVHD\textsuperscript{7} and radiation injury is not mandatory. Contraction of the total mTEC\textsuperscript{high} pool in unconditioned \(b \rightarrow bd\) recipients corresponded to progressive decreases in the subset which expresses Aire\textsuperscript{5,23} (Figure 1C-E). In 12-week old recipients, residual Aire\textsuperscript{*}mTEC\textsuperscript{high} were low in numbers (<300 cells/thymus) and later became virtually undetectable, owing to the smaller than normal frequencies of Aire-expressing cells among declining mTEC\textsuperscript{high} numbers.

Given the intimate associations between Aire-deficiency, TRA repression, and autoimmunity\textsuperscript{3}, we determined whether aGVHD interfered with TRA transcription. Development of thymic GVHD (\(b \rightarrow bd\)) altered global gene expression profiles in total residual mTEC\textsuperscript{high} cell pools isolated at 2-4 weeks after transplantation (Figure 2A, Table S1). Using a special algorithm (supplemental methods: "Bioinformatics") we discriminated between TRA and ubiquitously expressed transcripts (Ub) in mTEC\textsuperscript{high}. Several hundred TRA were present among transcripts whose expression levels were reduced by >3-fold in mice with aGVHD (Figure 2A, inserts). Notably, the TRA/Ub ratios for repressed transcripts were significantly higher than would have been expected to be observed by chance (O/E ratio >1.0 TRA; <1.0 Ub; \(p<0.001\); Figure 2A). A low correlation coefficient between TRA expression in \(b \rightarrow bd\) vs. \(bd \rightarrow bd\) mice (Pearson's \(r=0.700\) and \(r=0.625\) in 10 and 12 week old recipients, respectively) indicated a sizable number of TRA being reduced during lethal aGVHD (Figure 2B, Figure S3). We interpreted these data such that a contraction in TRA diversity during aGVHD was due to cellular loss of mTEC\textsuperscript{high}, in particular Aire-positive mTEC. This explanation was based on the fact that ectopic TRA expression is a stochastic
process where only a limited number of \( m\text{TEC}^{\text{high}} \) express a given TRA.\(^2,18\) A comprehensive coverage of TRA expression is therefore only achieved by a sufficiently large \( m\text{TEC}^{\text{high}} \) pool which is, however, missing under conditions of aGVHD. The most substantially reduced TRA were found to be enriched for genes that are characteristically expressed in tissues known to be targets of cGVHD\(^9\) (Figure 2C). Many of these genes belonged to the subset of Aire-dependent TRA\(^20\) (Figure 2D,F, Figure S3).

Since efficient central tolerance requires the full scope of TRA, we next asked whether \( m\text{TEC}^{\text{high}} \) compartment size and heterogeneity is sustained by employing strategies for epithelial cytoprotection. Peritransplant administration of the TEC mitogen Fgf7\(^15,16\) failed to prevent initial \( m\text{TEC}^{\text{high}} \) cell loss in \( b\to bd \) recipients (Figure 1A). However, Fgf7 maintained stable numbers of \( m\text{TEC}^{\text{high}} \), including the subset expressing Aire (~3000 cells/mouse during aGVHD; Figure 1C-E). Consistent with reports that mTEC are continuously replaced in adulthood,\(^18,22\) ~5% of mTECs underwent cell division within one week in the \( b\to bd \) and \( bd\to bd \) transplant groups (Figure 2E). This frequency increased to ~15% in mice with aGVHD but treated with Fgf7. Label retention analyses indicated high turnover of mTEC in response to Fgf7 in \( b\to bd \) recipients. Rescue of \( m\text{TEC}^{\text{high}} \) numbers was therefore the consequence of enhanced proliferation within the entire mTEC compartment. During early aGVHD the TRA spectrum was not preserved by Fgf7 (Pearson’s \( r=0.635 \) vs. \( bd\to bd \) mice; Figures 2B,S3). However, a broader array of TRA was expressed by 7 weeks after allo-HSCT as indicated by a expression profile that was closer to that of age-matched \( bd\to bd \) controls (\( r=0.807 \)). Hence, peritransplant administration of Fgf7 sustained a more diverse TRA transcriptome during aGVHD. Individual TRA expression patterns were differentially affected by aGVHD and Fgf7 as some but not all Aire-dependent TRA returned to nearly normal expression in the observation period (Figures 2F,S3,S4). However, the molecular mechanism for this biased profile remains to date unknown.
Taken together our data provide a mechanism for how autoimmunity may develop in the context of aGVHD (Figure S5). As thymic negative selection is sensitive to small changes in TRA expression\textsuperscript{4,5}, approaches for mTEC cytoprotection may prevent the emergence of thymus-dependent autoreactive T cells,\textsuperscript{12} and thus alter autoimmune outcome. The identification in cGVHD of the yet undefined specificities of autoreactive effector T cells\textsuperscript{24} will allow to test this argument directly in experimental systems and ultimately in clinical allo-HSCT.

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Authorship Contributions
S.D., G.N. and M.H.H. designed and performed work; R.I. implemented bioinformatics and statistics, W.K. and G.A.H. share senior authorship, and both designed work and wrote the paper.

Disclosure of Conflicts of Interest
The authors declare that there is no conflict of interest. There is also no non-author involvement in the preparation of a manuscript.

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References


Figure Legends

Figure 1. Acute GVHD impairs mTEC\textsuperscript{high} compartment size. The mTEC\textsuperscript{high} compartment was analyzed as a function of recipient age in four different murine models of allo-HSCT, as detailed in Figures S1 and S2. (A) Acute GVHD was induced in 8 week-old unconditioned BDF\textsubscript{1} recipients (b→bd , ■). Untransplanted BDF\textsubscript{1} mice (■) or syngeneically transplanted mice (bd→bd , ○) served as controls. In one group, Fgf7 was administered (b→bd + Fgf7 , □) as described.\textsuperscript{15} A total of 6 experiments were performed, with 3-5 mice/group and experiment. (B) Acute GVHD (TCD+T group, □) was induced in irradiated 8 week-old BDF\textsubscript{1} recipients (b→bd , left panel), 9 week-old B6 recipients (d→b , middle panel) or 8-week old B6 recipients (b→b , right panel) as described in the supplemental data. Untransplanted BDF\textsubscript{1} or B6 mice (■) and recipients of TCD bone marrow (TCD group, ○) were used as controls without disease in all models. A total of 3 experiments were performed. TCD, T-cell depleted; n.d., not done. The line graphs show total numbers (mean ± SD) of mTEC\textsuperscript{high} cells in thymi from individual mice. *p<0.05, ANOVA, aGVHD versus transplanted mice without aGVHD. (C,D,E) Qualitative and quantitative analysis of thymic Aire expression, as described in the supplemental methods. Immunohistochemistry and confocal microscope analysis (C) was performed on thymic frozen sections taken from unconditioned transplant recipients at ages of 10, 12 and 15 weeks (i.e. 2, 4 and 7 weeks after transplantation. Panels i-iii: bd→bd , iv-vi: b→bd , vii-ix: b→bd + Fgf7). Cytokeratin-18 (CK18, blue) and CK14-positive cells (red) denote cTEC and mTEC, respectively.\textsuperscript{14,17} Aire\textsuperscript{+} cells are in yellow color and localize to the thymus medulla. Syngeneically transplanted mice (bd→bd: 18±4 Aire\textsuperscript{+}mTEC/0.04 mm\textsuperscript{2} medulla) were not different from age-matched untransplanted control mice (data not shown and reference\textsuperscript{5}). The flow cytometry plots in (D) depict Aire and MHC class II expression of DAPI\textsuperscript{−}CD45\textsuperscript{−}EpCam\textsuperscript{−}Ly51\textsuperscript{−}UEA1\textsuperscript{+} mTECs. Numbers represent frequencies (%) of cells (mean ± SD). Total numbers of Aire\textsuperscript{+}mTEC\textsuperscript{high} in thymi from individual recipient mice (E) was calculated from flow...
cytometry data (mean ± SD). Groups are the same as in panel (A). A total of 5 experiments were performed, with 3-5 mice/group. *p<0.05, ANOVA, aGVHD versus transplanted mice without aGVHD.

Figure 2. Peritransplant administration of Fgf7 sustains a more diverse TRA transcriptome in mTEC\textsuperscript{high} during aGVHD. Acute GVHD ($b\rightarrow bd$) was induced in 8-week old unconditioned recipients BDF\textsubscript{1} mice as in Fig. 1A. Global gene expression was analyzed in entire residual mTEC\textsuperscript{high} cell pools isolated from individual recipients at 10, 12 and 15 weeks of age (i.e. 2, 4 and 7 weeks after transplantation). The figure reflects data from 5 independent experiments, with 2-4 mice/group and experiment. (A) Left panel: gene expression profiling of mTEC\textsuperscript{high} in transplant recipients using Mouse Gene 1.0 ST arrays (Affymetrix). The x- and y-axes represent relative expression of individual genes (given as log\textsubscript{2} values of signal intensity ratios, S.I.) in mice with aGVHD ($b\rightarrow bd$) versus syngeneically transplanted recipients without disease ($bd\rightarrow bd$). Data was plotted as a function of recipient age (10 vs. 12 weeks). Genes whose expression levels were enhanced or decreased by >3-fold relative to the control group (corresponding to relative expression values given by the signal intensity (S.I.) ratios of <0.33 and >3.0, respectively, or <-1.6 and >+1.6 on the log\textsubscript{2} scale) were considered to be significantly altered in consequence of acute GVHD (threshold is indicated by dashed lines). Q2 is the lower left quadrant, denoting genes whose expression levels were depressed at both 2 and 4 weeks after allo-HSCT (10 and 12 week old recipients). The inserted numbers denote the absolute numbers of genes in the respective quadrants. Transcripts from a total of 21,759 genes were analyzed. An annotated list of the genes most affected by aGVHD is given in Table S1. Ub (ubiquitously expressed genes, gray squares) and TRA (dark blue squares). Right panel: O/E performance analysis (O, observed number of events; E, expected number of events) of the presence of TRA versus Ub among the genes with relative expression <0.33 and >0.3. O/E ratio >1.0 for TRA and <1.0 for Ub; *p<0.001 using hypergeometric statistical analysis. A value of O/E =
1.0 represents a distribution among TRA and Ub according to expectations (that is sequestration according to the relative frequencies of TRA and Ub in adult mice without disease). (B) The linear relationship between two experimental groups was tested for Ub and TRA using a correlation matrix, as detailed in Figure S3. The variation of each gene was calculated using the interquartile range, a measure of statistical dispersion. Pearson’s correlation coefficient r is shown as a function of age and time after transplantation. The asterisk (*) symbol indicates a statistical p value for the quality of each measured r at any given time point for TRA and Ub, respectively; *p<0.001 of Pearson’s r. (C) Tissue representation of TRA repressed during aGVHD (<0.1 and <0.33 relative expression, respectively, versus mice without GVHD at 10 and 12 weeks of age). As control, a random set of 200 TRA was used that remained unchanged in the course of GVHD (relative expression ~1). Many repressed TRA were specific for tissues of the gastrointestinal tract, skin and eye. Changes in expression of these genes in response to Fgf7 are given in Figure S4. (D) Aire-dependency of TRA repressed in mTEC\textsuperscript{high} during acute GVHD in 10 and 12 week old recipients (quadrant Q2; the axes are the same as in panel A). Examples shown are the following: Scgb1a1, secretoglobin; Csn1s2a, casein \(\gamma\), Mup1, major urinary protein 1; Spt1, salivary protein 1 (see Table S1). Aire-dependent TRA (yellow circles), Aire-independent TRA (dark blue circles), Ub (gray circles). (E) Analysis of TEC proliferation in response to Fgf7. Eight week-old BDF\textsubscript{1} mice mice were left untreated or were treated with Fgf7 from days -3 to +3 after allo-HSCT. All mice simultaneously received 0.8 mg/ml BrdU in their drinking water. mTECs were analyzed for BrdU incorporation at the indicated ages after transplantation. BrdU\textsuperscript{+} cells (%) ± SD, *p<0.001, ANOVA, aGVHD versus mice without aGVHD. (F) Expression levels of individual genes expressed in mTEC\textsuperscript{high} is given in a heatmap as color coded log\textsubscript{2} value of signal intensities. Left panel: the map was sorted according to relative expression of genes in mTEC\textsuperscript{high} (\(b\rightarrow bd\) versus \(bd\rightarrow bd\) mice; 12 weeks old) and contains all TRA with a relative expression of <0.3 during aGVHD in age-matched mice. Enhancement of TRA expression in response
to Fgf7 was detectable by higher signal intensities of individual genes. Right panel: Aire-dependent TRA were sorted as above.
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