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Journal of Immunological Methods 329 (2008) 56–66

JIM
Journal of Immunological Methods

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Research paper

Unbiased analysis, enrichment and purification of thymic stromal cells

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Received 9 April 2007; received in revised form 21 September 2007; accepted 24 September 2007
Available online 23 October 2007

Abstract

The microenvironment of the thymus consists of functionally discrete niches composed of distinct stromal cell subsets. Clinically relevant changes affecting T-cell differentiation occur within these niches with age and injury caused by irradiation and chemotherapy treatments. The study of thymic stromal cells has been hampered by the technical difficulty in isolating significant numbers of this important population. Here we present an improved protocol for enzymatic isolation of stromal cells that enables comparative flow cytometric analyses and their purification for downstream cellular or molecular analysis. Fractions analyzed throughout enzymatic digestion of the thymus revealed that various stromal subsets are isolated at characteristic intervals. This highlights the importance of pooling all cells isolated from the thymus for numerical and phenotypic analysis to avoid biased representation of subpopulations. We also describe refined magnetic bead separation techniques that yield almost pure preparations of $CD45^-$ stroma. Sorting of these suspensions using defined markers enabled purification of the major epithelial subsets, confirmed by keratin staining and PCR analysis. This three-step procedure represents a rapid, reproducible method for the unbiased purification of the stromal cells that direct thymic T-cell differentiation.

Keywords: Thymus; Stromal cells; Purification; Epithelial cells; Flow cytometry

1. Introduction

T-cell differentiation in the thymus requires multiple, sustained interactions with different stromal cells. Thymocytes mature from double negative (DN, $CD4^-CD8^-$) precursors to double positive (DP, $CD4^+CD8^+$) cells in the cortex, and finally $CD4^+$ or $CD8^+$ single positives (SP) in the medulla. A variety of stromal cell subsets provide the signals that drive thymocyte differentiation, direct their migration through the thymus and impose central tolerance (Anderson and Jenkinson, 2001; Gray et al., 2005); however the precise molecular mechanisms underlying these processes are poorly understood. In addition, the lineage relationships between the epithelial subpopulations that establish the unique thymic microenvironments are yet to be defined. A major obstacle in studying thymic stromal cells has

Abbreviations: DN, double negative; DP, double positive; SP, single positive; TEC, thymic epithelial cell; TSC, thymic stromal cell; DC, dendritic cell.

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doi:10.1016/j.jim.2007.09.010

been their reproducible isolation for comparative analysis and enrichment in high numbers for purification for molecular and functional assays.

Despite their essential role, non-haemopoietic stromal cells are relatively rare, comprising less than 1% of thymic cellularity, and form an interconnected meshwork that further complicates their isolation. We previously refined methods for enzymatic isolation of $CD45^-$ stromal cells and described various markers that distinguish subpopulations by flow cytometry (Gray et al., 2002), however this technique alone does not provide sufficient enrichment to enable their rapid FACS purification for downstream analysis. Although enrichment techniques such as density gradients and magnetic bead separation have been employed in the past (Chidgey et al., 1998; Klein et al., 2000; Zuklys et al., 2000), loss of smaller stromal cells or viability reduces their applicability in certain experimental settings.

Here we describe improved methods for unbiased stromal cell analysis by flow cytometry and the purification of $CD45^-$ stromal cells using enzymatic digestion and automated magnetic bead depletion. These techniques isolate, enrich and maintain viability of all major $CD45^-$ stromal subsets, thus enabling more accurate comparative analyses. Thymic epithelial subpopulations were then sorted from these preparations in relatively high numbers, using various surface markers. Cells isolated in this way were analysed for expression of keratin and subset-specific molecules to confirm their type and purity. Together, these data define a rapid, reproducible method for enrichment of $CD45^-$ thymic stromal cells and their purification for functional and molecular analysis.

2. Materials and methods

2.1. Animals

C57BL/6 mice at 4–8 weeks of age were used for tissues in this study. Mice were bred and maintained at the Baker Institute Precinct Animal Centre or the Monash University Mouseworks Animal Facility according to institutional guidelines.

2.2. Antibodies and immunoconjugates

The conjugates used for flow cytometry were FITC-conjugated anti-CD45.2 (clone 104), FITC-conjugated UEA-1 lectin (Vector, U.S.A.), FITC-conjugated anti-CD8 (clone 53–6.7), FITC-conjugated anti-rat IgG (H+L) (Molecular Probes, U.S.A.), PE-conjugated anti-I-A/I-E (clone M5/114.15.2), PE-conjugated anti-CD4 (clone RM4-5), biotinylated anti-Ly51 (clone 6C3), biotinylated anti-CD31 (clone MEC13.3), PerCP Cy5.5-conjugated CD45 (clone 30-F11), APC-conjugated anti-TCRβ (clone H57-597), APC-conjugated anti-CD11b (clone M1/70), CyChrome-conjugated streptavidin, and APC-conjugated streptavidin, all purchased from Pharmingen (U.S.A.) unless otherwise stated. MTS-15 (Gray et al., 2007) and MTS-33 hybridoma supernatants were grown in the laboratory.

2.3. Individual thymus digestion for flow cytometric analysis

This procedure was adapted from a previously described protocol (Gray et al., 2002). Thymi were kept separate and digested individually. First, each thymus was thoroughly cleaned of fat and connective tissue, its lobes separated, and the capsule nicked with fine scissors. Thymi were then individually agitated in 10 ml of RPMI-1640 for several minutes to gently flush out as many thymocytes as possible. The supernatant was collected and replaced as it became visibly cloudy and kept on ice. Thymi were then incubated at 37 °C for 10 minutes in 2 mL of 0.125% (w/v) Collagenase D with 500 μL of 0.1% (w/v) DNAse I (both from Roche, Germany) in RPMI-1640, with regular, gentle agitation. Fragments were allowed to settle. The supernatant was collected, kept on ice, and the digestion repeated using the remaining settled thymic fragments. After 3 digestions, the remaining aggregates were incubated for 10 min, or until dispersed, with 3 mL of 0.125%(w/v) Collagenase/Dispase (Roche, Germany) with 300 μl of 0.1%(w/v) DNAse I in RPMI-1640. Cells from all supernatant fractions were then centrifuged at 472 $\times g_{max}$ for 5 min, pooled, and resuspended in cold EDTA/FACS buffer (5 mM EDTA in PBS with 2% FCS and 0.02% NaN_3). Cells were filtered through 100 μm mesh and cell counts were performed using a Z2 Coulter Counter (Beckman Coulter U.S.A.). For phenotypic analysis of stromal cells, 15×10^6 cells were stained.

2.4. Pooled thymus digestion for stromal cell isolation

Single cell suspensions of thymic stromal cells were isolated as previously described (Gray et al., 2002). Briefly, thymic lobes from 5, 10 or 20 mice were cleaned and prepared for digestion, and thymocytes flushed out as described in Section 2.3. Fragments were then resuspended in 5 ml of 0.125% (w/v) collagenase D and 500 μL of 0.1% (w/v) DNAse I (both from Roche, Germany) in RPMI and the mixture digested at