Lymphocytes in the Peritoneum Home to the Omentum and Are Activated by Resident Dendritic Cells

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The omentum is of interest in the context of obesity-related metabolic disease where adipose tissue exhibits inflammatory changes; however, the immunology of the omentum is underexplored. The greater omentum is draped from the stomach and consists predominantly of adipose tissue studded with lymphoreticular aggregations (milky spots) that distinguish it from other visceral adipose tissues. Milky spots are thought to contain and conduct leukocytes in transit from the blood to the peritoneal cavity, particularly during peritonitis. We show here that both B and T lymphocytes counterflow from the peritoneal cavity to the omentum in mice. Residence in the omentum was brief with a t½ residence time of 6 h. Omentum access was pertussis toxin-sensitive, dependent on activation of the Rap1 GTPase, and on the integrin LFA-1. B cells and CD44high T cells accessed the omentum most efficiently, but homing of resting CD44low T cells was also observed. Omental tissue from normal healthy mice was found to contain CD8⁺CD11bhighMHC class IIhighCD11chigh dendritic cells that promoted the rapid activation of T cells entering the omentum and cross-presented soluble OVA or OVA acquired from either OVA-expressing Escherichia coli or OVA-pulsed spleen cells. We conclude that the omentum incorporates two key features of immunological sentinel function, actively supported lymphocyte traffic and dendritic cells, that reinforce a conceptual framework for function in stimulating adaptive immunity. These results extend basic understanding of omental and peritoneal cavity immunology and of how proinflammatory events occurring within the peritoneal cavity might affect adipocyte and hepatocyte metabolism. The Journal of Immunology, 2009, 183: 1155–1165.

Most leukocytes found within the murine greater omentum are concentrated in milky spots, on the periphery of the adipose tissue band also known as the omental fat band (OFB). Milky spots are composed of both myeloid and lymphoid cells and are reactive structures that increase in size and number in response to peritoneal inflammatory stimuli. Milky spots develop a more organized structure where T and B cells segregate as in secondary lymphoid tissues (5, 6). This structural organization originally led to the proposal that milky spots were themselves secondary lymphoid organs (6). However, segregation of B and T cell areas was not observed in the resting omentum (5, 7). Furthermore, the failure to detect a significant number of dendritic cells (DCs) within the omentum (5, 7–9) led to the view that the greater omentum is an inflammation-induced lymphoid structure, lacking the defining characteristics of secondary lymphoid tissues including resident professional APCs, permanence in basic structure, and segregation of B and T cell regions in the absence of antigenic stimulation or inflammation.

Although there is precedent for omental absorption of protein and particulates from the PC, lymphatic drainage via this route is considered to be minor relative to exit via diaphragmatic and visceral lymphatics (10). Leukocytes may exit the PC upon resolution of an inflammatory response (11, 12) and, in response to innate immune activators, B-1 B cells migrate from the peritoneum to the spleen via several routes including the omentum (13). Recently, splenic B-2 B cells introduced into the PC were shown to access the omentum, but the molecular mechanisms involved were not identified (2). We report that when splenic or lymph node-derived T and B cells were introduced into the PC, they both accumulate rapidly and prominently in the OFB. These observations stimulated questions about the functional significance of such lymphocyte traffic, in particular for T cells. We therefore investigated the mechanisms underlying lymphocyte entry into the OFB, the distribution and...
dynamics of lymphocytes within the OFB, and the nature and functionality of APCs within the OFB.

Materials and Methods

Mice

Mice aged 7–16 wk were used for analyses. C57BL/6J (B6), BALB/c, CBA/J, H-Y (14), OT-1 (15), and OT-II (16) TCR transgenic mice were bred at the specific pathogen-free animal facility at the Biomedical Research Centre (University of British Columbia). Procedures employed in this study were approved by the Animal Care Committee at the University of British Columbia.

Media and buffers

Standard media was RPMI 1640 supplemented with 5% FCS, 5 × 10⁻⁵ M 2-ME, 100 U/ml penicillin, 100 U/ml streptomycin (StemCell Technologies), and 2 mM glutamine (Sigma-Aldrich). HBSS lacking Mg²⁺/Ca²⁺, 100 U/ml penicillin, 100 U/ml streptomycin (StemCell Technologies) was used for Ab blocking experiments.

Antibodies

Abs used in this study included CD8-allophycocyanin (BD Pharmingen, catalog no. 550355), CD4-PE (BD Pharmingen, catalog no. 553031), human IgG1-P-selectin fusion protein (BD Pharmingen, catalog no. 28111A) detected with PE-conjugated anti-human IgG (Jackson Immuno-Research Laboratories, catalog no. 109-116-098), CD8-biotin (Cedarlane Laboratories, catalog no. CL8933B) detected with CyChrome-conjugated streptavidin (BD Pharmingen, catalog no. 554062), PE-conjugated anti-αβ₂ chain DATK32 (BD Pharmingen, catalog no. 553811), anti-CD11b (Caltag Laboratories, catalog no. RM2004), anti-CD103 (BD Pharmingen, catalog no. 557495), 33D1 (eBioscience, catalog no. 12-5884-81), F4/80 (Caltag Laboratories, catalog no. MF48004), anti-Vv2 (BD Pharmingen, catalog no. 553289), and allophycocyanin-conjugated Gr-1 (eBioscience, catalog no. 17-5931-82), CD69 FITC (BD Pharmingen, catalog no. 553236), CD11c-biotin (BD Pharmingen, catalog no. 553800), anti-CD45-PE-B220 (BD Pharmingen, catalog no. 553900), anti-MHC class II (MHCI-β2m-FITC (eBioscience catalog no. 11-5321-85), and anti-CD4 GKL5 were also used. mAb T30.7 specific for male Ag (H-Y) specific TCR α-chain transgene product (17) was conjugated with biotin or Alexa Fluor 488. For blocking studies the following Abs were used: anti-α₄ integrin, rat IgG2b, clone F414.8; anti-β₂ integrin rat IgG1k, clone GAME46 (BD Pharmingen, catalog no. 555280); anti-α₄ integrin rat IgG2bk, clone P5/2 (Chemicon International, catalog no. CBL 1304); anti-β₂ integrin, hamster IgMk, clone 9B2/S (BD Pharmingen, catalog no. 555002); anti-α₂-β₃ integrin rat IgG2ak, clone DATK32 (eBioscience, catalog no. 16-5887-85); and control anti-CD45, rat IgG2b, clone I3/2.

Flow cytometer-based cell counting with beads

For cell counting, 100-μl aliquots of 10-μm latex beads at 10⁷/ml (2-1000; Interfacial Dynamics/Invitrogen) were mixed with an equal volume of cells and preincubated at 2 × 10⁹M Ca²⁺/Mg²⁺ in the omentum after i.p. injection. WEHI-231 cells were labeled with distinct tracking dyes, coinjected i.p., and compared for in vivo homing efficiency to the OFB 2 h later.

Imaging

Imaging details are provided in Extended Methods.

In vitro stimulations

Allogeneic mixed lymphocyte stimulations and primary HY-specific stimulations were conducted in 200-μl cultures plates (BD Falcon, catalog no. 353077) with 2000 rad-irradiated OFB walkout bulk cells, spleen cells, or DC-enriched fractions from spleen as previously described (22) with minor modifications (see Extended Methods). HY peptide KCSRNRQYL (23) was synthesized at the Biomedical Research Centre (University of British Columbia). OVA or HY peptide were included at 10 ng/ml or 100 μg/ml, respectively, during overnight preparation of DC-enriched fraction of spleen or OFB walkout stimulators. For allogeneic mixed lymphocyte reactions, responder lymph node cells were CFSE labeled and used at 2 × 10⁶/well. For anti-male Ag-specific reactions, CDB⁺ T cell enriched HY-TCR transgenic female donor lymph node cells were depleted of surface IgG⁺, CD4⁺ cells with anti-CD4 (GKL5), and sheep anti-mouse Ig-conjugated Dynabeads (Dynal Biotech/Invitrogen, catalog no. 110.31), and CFSE labeled.

Chelator inhibition of lymphocyte adhesion to omental explants

Peripheral lymphocytes from spleen and lymph nodes were harvested in RPMI 1640 containing 0.5% BSA and 10 mM HEPES, labeled with CFSE, and preincubated at 2 × 10⁷/ml for 45 min at 37°C with rotation. Cells were filtered through cotton wool and combined with fresh OFB explants in the same media with or without 5 mM chelators indicated for 20 min with rolling. OFB were then rinsed in the same media, Hanks (+), and then imaged for bound CFSE-labeled cells.

Integrin blocking analyses

Competitive in vivo assays were developed to assess anti-integrin mAb inhibition of lymphocyte accumulation in the OFB. Details are provided in Extended Methods. Briefly, aliquots of donor peripheral lymphocytes distinguished with distinct tracking dye labels were treated with anti-integrin or control mAbs and coinjected i.p. for competitive OFB homing. Fifty minutes later, OFBs were harvested and donor T and B cells were recovered by the overnight walkout procedure and enumerated. Three replicate samples of 1000 donor cells from input, OFB, or PC samples were collected by flow cytometry and the ratios of cell yields were determined for anti-integrin pretreated/no Ab control and for isotype control pretreated/no Ab control groups.

Cross-presentation

Cross-presentation was demonstrated with three distinct preparations of OFB walkout cells. OFB walkout cells were prepared in the presence of 10 mg/ml soluble OVA, washed, and irradiated with 2000 rad. Alternatively, mice received an i.p. injection of a PBS-washed 0.3-ml sample of stationary phase control DH5α Escherichia coli or DH5α transformed with pUC18

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The online version of the article contains Extended Methods.
plasmid and expressing an OVA cDNA insert (provided by Dr. M. J. Wick, Göteborg University, Göteborg, Sweden). Two hours later, OFBs were explanted and walkout cells prepared in the presence of 50 μg/ml gentamicin-supplemented culture media. OFB walkout cells were then irradiated (2000 rad) for use as stimulators. Lastly, BALB/c spleen cells were harvested in serum-free RPMI 1640, pulsed for 10 min in RPMI 1640 including 10 mg/ml OVA at 37°C, irradiated (1500 rad), and washed three times before i.p. injection of B6 recipients. Four hours later OFBs were harvested, rinsed, and cultured for overnight walkout cells. OFB walkout cells were then irradiated (2000 rad) for use as stimulators. These three walkout preparations were used to stimulate CFSE-labeled OT-I lymph node cells (3 x 10^7/well) for 3 days when proliferation (CFSE dilution) of viable OVA-specific cells bearing the relevant CD8 or CD4 co-receptor and transgenic Vα2 TCR was assessed.

Results

Peritoneal lymphocytes migrate into the OFB

Short-term tracking studies with lymphocytes labeled with CFSE or expressing a GFP transgene revealed that donor lymphocytes injected into the PC accumulated rapidly (1–2 h) in the OFB (Fig. 1A). The total numbers of donor cells recovered from the OFB remained roughly constant at 10^7 cells per mouse and were evident at least as long as donor cells persisted in the PC (Fig. 1B). One early, immunologically relevant destination of lymphocytes after i.p. injection is reported to be the pancreatic lymph node where relatively large numbers of donor lymphocytes accumulate within 20 h and can respond to Ag (24). When plotted in terms of either absolute numbers of donor cells or percentage of donor cells among total lymphocytes recovered for various tissues, the pancreatic lymph node did contain an abundance of donor cells relative to other nodes but was a minor destination when compared with the OFB (Fig. 1C). Significant PC drainage to the parathympic lymph nodes has also been described (12). However, at the time point assessed, the total donor lymphocyte recovery per mouse from the four pooled parathympic nodes was comparable to that seen for mesenteric nodes (data not shown). Based on confocal microscopic analysis, it was recently reported that 24 h after i.p. adoptive transfer of spleen cells, comprised of both T cells and B cells, only B220+ donor splenic B-2 B cells were detected in recipient omenta (2). We evaluated short-term (5 h) lymphocyte homing efficiency to the OFB as assessed by recovery of donor cells after overnight OFB walkout as described in Materials and Methods. As shown in Fig. 1D, and consistent with these observations, B cells exhibited preferential accumulation in the OFB. However, donor T cell accumulation clearly occurred as well. CD8+CD44high T cells (Fig. 1E) (and CD4+CD44high T cells; data not shown) accumulated preferentially in the OFB relative to their naive CD44low counterparts. Thus, all of the major lymphocyte subsets that we assessed clearly exhibited OFB homing ability.

Lymphocyte dynamics in the OFB

The experiments described above established that lymphocytes introduced into the PC accumulate and persist in the OFB. Whether this persistence reflected the filling and static residence of donor cells or the filling and ongoing exchange of donor cells from the PC was not resolved. To gain a better understanding of lymphocyte dynamics in the OFB, an in situ labeling technique was developed. Briefly, mice given an i.p. injection of CFSE-labeled donor lymphocytes were rested overnight and then received a second i.p. injection with CellTracker Orange (CTO). As shown in Fig. 2A, CTO did not penetrate well into deeper areas of the OFB, resulting in CFSE−CTOvariable donor cells in the OFB and CFSE+CTOhigh donor cells in the PC immediately after injection. The equilibration of CFSE+CTOhigh cells in the peritoneal and OFB compartments reflected lymphocyte turnover in the OFB (see Extended Methods for technical details). The kinetics of equilibration of these compartments was best approximated by an exponential curve shown in Fig. 2B. Regression analysis of this curve gave a correlation coefficient of 0.9634, yielding t_{1/2} estimates of 5.3 and 6.3 h at 45% and 40% values for %CTOhigh PC minus %CTOhigh OFB, respectively.

FIGURE 1. Lymphocytes accumulate in the OFB after i.p. injection. A, Light and fluorescence image of OFB 4 h after i.p. injection of 10^7 GFP+ donor lymphocytes (1 mm scale bar). B, Donor cells recovered from recipient PC (filled bars) or mechanically dissociated OFB (open bars) after i.p. injection of CFSE-labeled lymph node and spleen cells. C, Recovery of donor CFSE-labeled surface Ig+ (lymph node and spleen) cells from the OFB and CFSE+ lymphoid organs 20 h after i.p. injection. Data are expressed as absolute numbers of CFSE+ cells per organ (left panel) or percentage total lymphocytes per organ (right panel) from brachial (Br), pancreatic (Pan), and mesenteric (Mes) lymph nodes, or from OFB walkout cells (OFB). D, Donor spleen and lymph node cells were marked with 0.1 and 1.0 μM CFSE, respectively, and pooled before i.p. injection. Percentages of donor T cells (dark blue bars) vs B cells (light blue bars) in input cells (in) and recovered from the PC (P) or from OFB walkout (O; see Materials and Methods) from four recipients 5 h after i.p. injection of 1.5 x 10^7 donor cells are shown. E, Same as D except that recovery of naive CD44low/CD8+ (dark green bars) vs memory CD44high/CD8+ T cells (light green bars) is shown.
To assess whether resident peritoneal lymphocytes normally access the OFB, a similar in situ CTO labeling method was applied where mice received a single i.p. bolus of CTO labeling solution to mark all free peritoneal cells and exposed OFB cells as described in Fig. 2A. Mice were then sacrificed 4 or 9 h later. As shown in Fig. 2C, when cells were isolated from the OFB at 9 h, the proportion of T cells labeling intensely with CTO increased relative to the 4 h time point. If the normal flow of T lymphocytes was unidirectional from the OFB out into the PC, the proportion of CTO<sup>high</sup> cells extracted from the OFB should have decreased with time after the CTO injection. Since this did not occur, the results provide the first evidence for resident T lymphocyte traffic from PC to the OFB.

**T/B segregation in nonactivated OFB**

It was previously reported that T and B cells segregate within the OFB only under conditions of inflammation (5, 7, 25). When differentially labeled T and B cells were introduced into the PC, cell clusters of varying sizes accumulated within the OFB. When the clusters were small or diffuse, the donor cells did not exhibit clear T/B lineage segregation. However, instances were observed where a layered organization appeared to be present, with T cells being deeper and B cells more superficial (Fig. 3). These results suggested that lineage segregation can occur, perhaps only at specific sites where sufficient numbers of lymphoid cells had colocalized.

**Mechanisms of lymphocyte adhesion in the OFB**

Little is known about the molecular mechanisms responsible for lymphocyte homing to the OFB. Lymphocytes lacking L-selectin accumulated in the OFB to the same extent as L-selectin<sup>+/−</sup> lymphocytes (data not shown), arguing against a role for selectins in this process. However, consistent with results reported for B-1 B cells (13), pretreating donor peripheral lymphocytes with pertussis toxin inhibited CD8<sup>+</sup> T cell (Fig. 4A), CD4<sup>+</sup> T cell (data not shown), and B-2 B cell (data not shown) accumulation in the OFB. The partial inhibition of OFB homing noted in Fig. 4A raised the question of whether naive or memory CD8 T cell homing to the OFB was differentially susceptible to pertussis toxin. We therefore assessed recovery of CD44<sup>high</sup> (memory) vs CD44<sup>low</sup> (naive) CD8<sup>+</sup> T cells from the OFB after pertussis toxin treatment; both naive and memory subsets were sensitive to pertussis toxin, although naive T cells appeared to be marginally more sensitive (data not shown).

The data shown in Fig. 4A clearly demonstrate that OFB access within the brief 2-h assay is pertussis toxin dependent. The interpretation of residual binding was not addressed, but visual inspection of OFB exposed to tracking dye-labeled untreated vs pertussis toxin-treated cells suggested that the latter adhered superficially to the OFB whereas untreated cells entered the OFB.

Chemokine-induced integrin activation plays a key role in the entry of lymphocytes into secondary lymphoid organs. Previous work has shown that chemokine-induced B cell migration, as well as chemokine-induced activation of the LFA-1 and VLA-4...
integrins in B cells, is dependent on activation of the Rap GTPases (26, 27). These processes are effectively inhibited when Rap activation is blocked in B cell lines (e.g., WEHI-231) by expressing the Rap-specific GAP protein Rap-GAPII. Rap-GAPII converts the Rap GTPases to their inactive GDP-bound form while having no effect on other signaling pathways or on the activation of the Ras, Rac, or RhoA GTPases (Ref. 27 and references therein). Competitive homing assays showed that

![Figure 4](https://example.com/figure4.png)

**FIGURE 4.** Lymphocyte adhesion to the OFB. A, Donor peripheral lymphocytes were labeled with 0.05 μM CFSE (CFSELow) or 0.5 μM CFSE (CFSEHigh). Where indicated, CFSELow cells were also pretreated with pertussis toxin (*). CFSEHigh and CFSELow cells were combined in equal numbers and injected i.v. or i.p. as indicated. Ratios of CFSELow/CFSEHigh among injected (input) cell suspensions (IN) is indicated. Two hours later, peripheral blood (B), peripheral lymph node (brachial, axillary, and submandibular) (L), free cells in the PC (P), or cells mechanically liberated from the OFB (O) were isolated and stained for CD8 and CD4 expression. Pertussis toxin interference with lymph node homing and OFB homing was reflected by reduced ratios of CFSELow/CFSEHigh cells recovered from these tissues. Data shown are for CD8+ T cells, but similar results were obtained for CD4+ T cells and B cells (data not shown). Results were qualitatively reproduced in two additional experiments. B, Equal numbers (10^7) of WEHI-231 cells transfected with empty vector (W) or Rap-GAPII-encoding vector (Rap-GAPII) were labeled with CFSE or CTO and injected i.p. After 2 h, free peritoneal cells (PC) contained approximately equal numbers of both cell types (top panels). Bottom panels, Fluorescence images of the OFB (100 μm scale bar shown). Right panels are the same as left panels except dyes were reversed. Results were qualitatively reproduced in two additional experiments. C, In vitro binding of CFSE-labeled lymphocytes to OFB explants blocked by EDTA but not EGTA (1 mm scale bar is shown). D, Anti-LFA-1 mAbs inhibit B cell and T cell access to the OFB. Data are expressed as the ratio of cells precoated with single mAbs specific for integrins (αLβ2, α4β1, α4β7), control anti-CD45 (45), or untreated cells (NA) vs numbers of untreated cells recovered from the PC (P) or the OFB (O). Left panels, All data points obtained from four separate experiments; right panels, corresponding histograms showing respective means and SDs.
omental accumulation of control WEHI-231 cells was substantially greater than Rap-GAPII-expressing cells 2 h after i.p. coinjection (Fig. 4B). This suggests that homing of B lymphocytes to the OFB is an active process requiring signal transduction through Rap GTPases.

Further evidence for integrin involvement in lymphocyte homing to the OFB was obtained through analysis of divalent cation requirements. Chelating Ca\(^{2+}\), Mg\(^{2+}\), and Mn\(^{2+}\) ions with EDTA prevents adhesion to integrin ligands, but removal of Ca\(^{2+}\) alone with EGTA is tolerated or even improves adhesion. As shown in Fig. 4C, in vitro binding of lymphocytes to OFB explants was effectively blocked by EDTA, but not by EGTA, consistent with integrin-mediated adhesion.

To identify integrins relevant for the OFB homing process, donor cell populations distinguished by different tracking dyes were preincubated with blocking mAbs to various integrin subunits expressed on normal resting lymphocytes. Competitive short-term OFB homing assays were performed after i.p. injection of control untreated donor cells and Ab pretreated donor cells. As shown in Fig. 4D, mAbs against either the $\alpha_4$ or $\beta_2$ integrin subunits significantly reduced the recovery donor derived T and B cells from the OFB (Student’s t test p values: for T cells, $\alpha_4 = 5 \times 10^{-13}$, $\beta_2 = 2 \times 10^{-12}$; for B cells, $\alpha_L = 4 \times 10^{-11}$, $\beta_2 = 2 \times 10^{-12}$), whereas mAbs specific for $\alpha_L$, $\beta_1$, and $\alpha_4\beta_2$ integrins or anti-CD45 isotype control did not. Surprisingly, the anti-CD45 mAb appeared to enhance B cell, but not T cell, entry into the OFB. This observation was provocative but difficult to interpret based on current understanding of CD45 function. These results demonstrated that the $\alpha_4\beta_2$ (LFA-1) integrin participated in B and T lymphocyte homing to the OFB.

![Figure 5](http://www.jimmunol.org/Downloadedfrom/1160_PERITONEAL_LYMPHOCYTE_HOMING_TO_OMENTAL_DCs)

**FIGURE 5.** Activated T cells appear rapidly in the OFB. A, CFSE-labeled peripheral T cells (CD8\(^{+}\) T cell enriched) from male mice expressing the male Ag-specific TCR transgene were injected i.p. into male or female recipients and recovered from the OFB or PC 9 h later. CD69 expression is shown for CD8\(^{+}\)T3.70\(^{+}\) male Ag-specific cells (thin line) and male Ag-nonreactive CD8\(^{+}\)T3.70\(^{+}\) donor cells (bold line). B, Same as in A except that forward light scatter was assessed in male Ag-specific CD8\(^{+}\)T3.70\(^{+}\) T cells at 28 h. C, Same as in A except that male recipients received CFSE\(^{+}\) donor CD8\(^{+}\) T cells i.v. (closed symbols) or i.p. (open symbols). Donor cells were then recovered on day 3 from tissues indicated; PerLN, brachial and axillary lymph node; PanLN, pancreatic lymph node; PC, peritoneal cells. The geometric means of P-selectin ligand expression is shown for responding T cells (CFSE diluted) CD8\(^{+}\) T3.70\(^{+}\) male Ag-specific T cells. When cell numbers per division fell below 10% of total, geometric means of staining were not included in graph. Qualitatively similar results were observed in two additional experiments. D, Allosstimulatory capacity of OFB walkout cells was assessed with lymph node cell responders and stimulator cells (stim) obtained from either B6 or CBA mice. CFSE-labeled B6 lymph node responder cells ($2 \times 10^5$) were mixed with specified numbers of irradiated (2000 rad) syngeneic (control) or allogeneic whole spleen cells (Spl), splenic DC-enriched plastic-adherent fraction (Spl DC), or OFB walkout cells. Four days later, the proliferative response (dilution of CFSE) was assessed. Data shown are for one of three qualitatively consistent experiments. E, Stimulatory capacity of OFB walkout cells was assessed with CD8\(^{+}\) male Ag-specific T cells and male peptide. The plastic-adherent fraction of spleen cells and the OFB walkout cells prepared with or without 100 $\mu$g/ml HY peptide were irradiated (2000 rad) and cultured with CFSE-labeled male Ag-specific CD8\(^{+}\) lymph node T cells. Proliferation of male Ag-reactive CD8\(^{+}\)T3.70\(^{+}\) T cells was assessed on day 3 by CFSE dilution. Responder numbers per well are indicated over stimulator numbers initially cultured for each row of histograms. Data shown are for one of four consistent experiments. F, CFSE-labeled HY lymph node cells were stimulated for 4 days with splenic DC or OFB walkout APCs preloaded with male peptide. Expression of P-selectin ligand and CD8\(^{+}\) male Ag-nonreactive CD8\(^{+}\)T3.70\(^{+}\) donor cells (bold line).
The OFB is a site of rapid T cell activation

Male Ag-specific T cells from HY TCR transgenic mice express high levels of both CD8 and the TCR transgene as indicated by staining with mAb T3.70<sup>high</sup>. Unlike polyclonal peripheral T cells that consist of both naive CD44<sup>low</sup> and memory CD44<sup>high</sup> subsets, these male Ag-specific T3.70<sup>high</sup> CD8<sup>+</sup> T cells are uniformly naive when isolated from their HY TCR transgenic female transgenic donors (28). Moreover, these cells retain their naive CD44<sup>low</sup> phenotype upon adoptive transfer into lymphopenic recipients in the absence of male Ag (29, 30). When lymphocytes from female HY TCR transgenic mice were introduced into the PC of male mice, most male Ag (HY)-specific CD8<sup>+</sup> T cells present in the OFB 9 h after injection had up-regulated CD69 expression (Fig. 5A). At this time point there was little if any evidence of CD69 up-regulation among free peritoneal male-specific CD8 T cells. At 28 h postinjection (Fig. 5B), blastogenesis was evident in peritoneal male Ag-specific cells but more so in OFB-associated cells. By day 3 postinjection, CD69<sup>high</sup> cells were occasionally present, as exemplified in the data set shown, among free peritoneal male-specific CD8 T cells. The rapid onset of CD69 expression described above indicated that Ag-presenting DCs might be present in the omentum, although previous studies have suggested that the omentum lacks DCs (8) based on both phenotypic (5, 9) and functional criteria (20). We therefore assessed the Ag presentation capabilities of OFB resident cells for their ability to activate naïve allogeneic or male peptide-specific T cells. As shown in Fig. 5D, cells recovered after overnight OFB walkout exhibited significant Ag presentation activity as measured by the stimulation of proliferation (CFSE dilution) of allogeneic but not syngeneic T cells.

Male peptide-specific Ag-presentation activity was also evident with peptide-loaded OFB walkout cells (Fig. 5E). Male Ag-specific TCR transgenic CD8<sup>+</sup> T cells responding to male peptide-loaded spleen DC-enriched stimulators expressed relatively high levels of P-selectin ligand but negligible α<sub>β</sub> integrin, whereas those responding to OFB walkout stimulators expressed α<sub>β</sub> integrin but little if any P-selectin ligand (Fig. 5F). These data establish that the OFB contains APCs that stimulate naïve alloantigen-specific T cells and differentially imprint homing receptor expression relative to splenic DCs.

Phenotype of APCs of the OFB

To characterize the OFB-derived APCs, cell surface phenotyping was performed on OFB walkout cells. Male peptide-loaded OFB walkout cells were sorted into four fractions based on staining with B220, MHC-II, and CD11c and then assayed for the ability to stimulate male peptide-specific T cells as shown in Fig. 6A. Fraction 4, consisting of MHC-II<sup>high</sup>B220<sup>low</sup>CD11c<sup>high</sup> cells, exhibited the stimulatory and phenotypic hallmarks of DCs.

Cell surface phenotyping was performed on OFB walkout DCs as shown in Fig. 6B. Based on forward light scatter (FSC) properties, these cells were large (R2) relative to resting lymphocytes (R1). OFB DCs were resolved (region R4) after gating for viable (propidium iodide-negative) FSC<sup>high</sup>CD19<sup>−</sup>CD11c<sup>high</sup>MHC-II<sup>high</sup> cells. These cells represented ~1% of OFB walkout cells. Walkout cell yields for 8- to 10-wk-old mice were 145 ± 75 x 10<sup>7</sup> (n = 8) with higher yields in older mice and 6- to 8-fold higher in 18-wk-old mice. Staining of these R2/R3/R4-gated cells for various cell surface markers indicated that they were relatively homogeneous. Minor subpopulations of CD4<sup>+</sup> and CD103<sup>+</sup> DCs were occasionally present, as exemplified in the data set shown, or CD4 co-receptors per well is shown for indicated doses of irradiated splenic DC-enriched stimulators per well.

**FIGURE 6.** Phenotype of APCs of the OFB. A, OFB walkout cells prepared with and without male peptide were sorted into four fractions (1–4) according to the dot plot, and used in parallel with unfractionated OFB and control spleen (Spl) DC-enriched fractions to stimulate CFSE-labeled CD8<sup>+</sup> lymph node T cells from male Ag-specific TCR transgenic female mice. Histograms on the right side of the figure show CFSE fluorescence profiles for male Ag-specific CD8<sup>+</sup>T3.70<sup>high</sup> cells in cultures stimulated with each fraction on day 4. B, OFB DCs were effectively resolved from nonstimulatory OFB walkout cells by gating on propidium iodide- (PI) negative, CD19<sup>−</sup> cells with a MHC-II<sup>high</sup>CD11c<sup>high</sup> phenotype. Cell surface staining of these OFB DCs with additional mAbs is shown in histograms on right. C, Stimulatory efficiency of sorted CD11c<sup>high</sup>MHC-II<sup>high</sup>CD11b<sup>high</sup>OVB walkout cells prepared in the presence of soluble OVA was compared with unsorted OFB or OFB walkout cells depleted of these cells. Lymph node responder cells (3 x 10<sup>4</sup>/well) were from either OT-I mice (class I MHC-restricted CD8<sup>+</sup> T cells; left panel) or OT-II mice (class II MHC-restricted CD4<sup>+</sup> T cells; right panel). Numbers of proliferating (CFSE diluted) Vα2 responder cells bearing the appropriate CD8 or CD4 co-receptors per well is shown for indicated doses of irradiated stimulators per well.
but DCs with a CD8\(^{-}\), CD11b\(^{high}\), MHC-II\(^{high}\), F4/80\(^{-}\), 33D1\(^{-}\), CD4\(^{-}\), CD103\(^{-}\), CD45\(^{+}\) phenotype were heavily predominant and consistently present. These cells did not express the Ly6c(Gr-1) marker (data not shown). F4/80\(^{+}\) cells with a CD11c\(^{low}\) phenotype were also present in OFB walkout cells and fell within the high 90\(^{\circ}\) side light scatter gate R5 (discussed below).

Stimulator cell titration shown in Fig. 6C revealed relatively potent Ag presentation activity for OVA-specific OT-I CD8\(^{+}\) T cells by purified CD11c\(^{high}\)MHCII\(^{high}\)CD11b\(^{high}\) cells, whereas depletion of this subpopulation from OFB walkout cells yielded a fraction largely devoid of stimulatory function.

**Cross-presentation by OFB DCs**

The ability of OFB walkout DCs to process soluble OVA and activate naïve class I MHC restricted OT-I T cells shown in Figs. 6C and 7A demonstrated that these cells could cross-present exogenous Ag. The cross-presenting activity of OFB DCs was unexpected since these cells exhibit a CD8\(^{-}\)CD11b\(^{high}\) phenotype, contrasting with the CD8\(^{-}\)CD11b\(^{+}\) phenotype generally associated with cross-presenting DCs in secondary lymphoid tissues (31, 32). However, it was possible that the high dose of soluble OVA used in these experiments enabled cross-presentation in DCs that would otherwise not function in this way (33). We therefore explored more relevant and rigorous methods for evidence of cross-presenting activity. As shown in Fig. 7, OFB walkout cells could process and cross-present OVA after i.p. exposure to either OVA-expressing E. coli (Fig. 7, A and B) or irradiated and OVA-pulsed MHC-mismatched BALB/c spleen cells (Fig. 7C). Thus, OFB DCs with a predominant CD8\(^{-}\)CD11c\(^{high}\)MHC-II\(^{high}\)CD11b\(^{high}\) phenotype can cross-present exogenous Ag for class I MHC-restricted T cell responses.

**Discussion**

The greater omentum is generally regarded as an obscure structure whose status as an organ is uncertain on grounds that its function is poorly defined. The limited immunological interest in the omentum stems from the understanding that it lacks DCs, lacks stable lymphoid organization, and simply routes leukocytes into the peritoneal cavity.

The omentum has long been known to be an adsorbent of foreign particulates in the peritoneal fluid (Ref. 34 and references therein) and as such may sample, over a lifetime, the contents of the peritoneal cavity that houses the entire gut. The omentum, particularly the milky spots, contain a range of more or less mature cell types including macrophages, T, B1, and B2 lymphocytes, mast cells, and DCs (this report) and is recognized as a highly reactive structure in outright peritonitis. The greater omentum predominantly consists of adipocytes and is considered to be one of several visceral adipose tissue depots. The relationship between adipose tissue and lymphoid tissue is more complex than simply energy production by the former and consumption by the latter (35). Immune responses can promote adipose tissue hypertrophy in both adjacent and distant depots (Refs. 35, 36 and references therein), and adipose tissue can produce adipokines that include potent proinflammatory mediators (37). The fundamental importance of this interplay between immunological tissue and adipose tissue has recently emerged in concepts about the inflammatory basis of the metabolic syndrome (37–43), a constellation of clinically serious comorbidities of obesity including atherosclerosis, hypertension, hyperlipidemia, and type 2 diabetes. Visceral obesity is a good correlate of metabolic syndrome (44) and proinflammatory dialogue between lymphomyeloid cells and adipocytes (35), especially in visceral adipose tissues (44) including the omentum (45, 46), is thought to participate in the metabolic disturbances associated with obesity (37–39). Immunological activity in the OFB may thus be of importance in the context of metabolic disease. Interestingly, despite subanatomical similarities, in proportion to total body weight, human greater omenta (0.3–2 kg) are ~10-fold larger than murine greater omenta (10–40 mg).
The observations we report herein advance understanding of omentum immunology in several significant ways. In broad terms these include flow of B and T lymphocytes from PC to the OFB via an active homing mechanism, lymphocyte dynamics within the omentum, early T cell activation in the omentum in the presence of cognate Ag, and the identification of omental DCs able to cross-present exogenous Ag.

Several specific insights into the mechanisms underlying lymphocyte homing to the OFB were achieved. This homing was demonstrated experimentally with both T cells and B cells after adoptive transfer of donor cells but was also demonstrated for resident peritoneal T lymphocytes. It was recently reported that, based on confocal microscopic analysis, adaptively transferred lymphocyte access to OFB from the PC was restricted to B-2 B cells, as T cells were apparently excluded (2). In contrast, we found that naive CD44low CD4+ and CD8+ T cells readily enter the OFB, although our data indicate that naive T cells enter the OFB somewhat less efficiently than do B cells or CD44high memory T cells.

The relative recoveries of B cells, CD44low cells, and CD44high cells generated by mechanical means or by overnight walkout were similar (see Materials and Methods), indicating that these leukocyte subpopulations exit the OFB comparably during the overnight walkout process. Furthermore, the relative proportions of the major leukocyte subpopulations we observed after overnight walkout from unmanipulated OFB are in good agreement with those recently reported for collagenase digests of OFB (47) with the exception that F4/80+ macrophages appear to exit inefficiently in the walkout process (data not shown). The apparent differences in homing efficiency between naive and memory cells as noted presumably reflect differences in chemokine responsiveness (see below).

Lymphocyte traffic into the OFB appears to be an active process requiring cell signaling, as OFB entry of the B cell line WEHI-231 was profoundly dependent on Rap activity. Rap1 activation is a major step in chemokine-induced lymphocyte migration and integrin activation (48). The impact of Rap inactivation on WEHI-231 cells presumably reflects its role in reorganizing the actin cytoskeleton during chemokine-induced migration as well as its critical role in the inside-out signaling that leads to integrin activation. We have shown previously that CXCL13 activates Rap in B cells, such as WEHI-231, and that Rap activation is required for CXCL13 to stimulate B cell migration and integrin-mediated adhesion (27).

T (naive and memory) and B lymphocyte homing to the OFB was also inhibited by pertussis toxin, thereby implicating Gαi subunits used in signaling by various receptors including the homeostatic chemokines CCL19, CCL21, CXCL13, or CXCL12 (13, 49). The chemokine CXCL13 is produced in the omentum and supports B-1 B cell entry (1). However, since resting T cells lack CCR5 expression, their entry into the OFB cannot utilize omental CXCL13. Therefore, pertussis toxin inhibition of B and T cell accumulation in the OFB reinforces the view that this homing is an active process supported in both major lymphocyte subsets and suggests that another chemokine (possibly CXCL12/SDF-1) supports T lymphocyte homing to the OFB. Preliminary attempts to block OFB homing with CXCR4-specific mAbs (2B11), inhibitors (AMD3100), and an SDF-1 peptide antagonist were unsuccessful.

Cation-chelator and Ab-blocking experiments implicated the integrins, specifically α4β1 (LFA-1), in B and T lymphocyte homing to the OFB. A prominent role for α4β2 integrin (LFA-1) in OFB homing is consistent with the broad range of lymphocytes that can access the OFB from the PC, as this integrin is expressed on all lymphocyte populations that we assessed for OFB homing. It is intriguing that three different integrins appear to regulate lymphocyte traffic through the omentum: the integrin α4β1 controls blood-to-omentum flow (2), α4β1 controls omentum-to-PC flow (2), and α4β2 controls PC-to-omentum flow (this study). It will be of interest to identify the nature of the distinct upstream signals that coordinate usage of these integrins.

The dynamics of lymphocyte turnover after OFB entry was also explored. This was achievable due to the compartmentalization of the OFB within the PC and the use of an in situ labeling method that labels free cells in the PC with limited OFB penetration. Limits on tracking dye penetration within microvasculature has been demonstrated and exploited to mark proximal vascular trees (50). Under the experimental conditions used, the turnover of lymphocytes within the OFB was surprisingly rapid; approximately half of donor lymphocytes within the OFB are replaced with recent immigrants from the PC within 6 h. This analysis is the first to document cell turnover dynamics in the omentum to our knowledge.

The methods we have applied do not resolve whether the PC-to-OFB lymphocyte traffic is generally accompanied by flow in the reverse direction, but recent results suggest that this is indeed the case (2). Whether the transient nature of lymphocyte residence within the OFB reflects omental function as a peritoneal exit route, as a terminal destination, or as a local cavity-restricted trafficking pathway where lymphocytes recirculate between the PC and OFB remains to be determined. Clarification of this issue will be technically difficult but helpful in revealing how lymphocyte flow to the OFB contributes to the immune response in relationship to proximal secondary lymphoid tissues.

Regardless of their ultimate fate under resting conditions, lymphocytes accessing the OFB will encounter myeloid cells known to reside there. In vivo, and in the presence of Ag, we found that early activation markers, CD69 coupled with blastogenesis, were prominent on Ag-specific T cells within the OFB relative to free cells of the PC, suggesting that under appropriate conditions the OFB may effectively operate as an early and local site for Ag presentation; our finding that cells with phenotypic and functional properties of DCs can be readily isolated from murine OFB was also consistent with this scenario. We demonstrated that cells emigrating from resting murine omentum after overnight organ culture could activate both alloantigen-specific and peptide-specific T cells in otherwise unsupplemented cultures. Such stimulation differentially imprints responding T cells for αβ2 expression, but not P-selectin ligand expression, with the former being one marker of gut-specific recruitment potential. These stimulatory CD11c+ MHC-IIhigh/CD19/B220+ cells therefore exhibit both functional and phenotypic hallmarks of conventional DCs (51, 52). DCs were recently identified in older human omenta (17 adult donors aged 25–69 years, mean age of 53 years) (19), but walkout cells from omenta of 6- to 8-wk-old mice reportedly failed to stimulate in mixed lymphocyte reactions despite exhibiting some phenotypic properties of DCs (20). This discrepancy with our observations might have occurred if macrophages, or other myeloid cells with inhibitory activity in mixed lymphocyte reactions, were more prevalent in OFB of mice used in the latter study relative to mice used in our study. Another possible explanation is that OFBs from mice we used were in an activated state and thus harbored an inordinate DC content. This possibility seems unlikely as our colony is specific-pathogen-free and OFB-derived stimulatory activity was reliably demonstrable in mice 7 wk of age (youngest tested) and older. It was recently reported that 4% of omental cells liberated with collagenase exhibited a CD45+CD11c+ phenotype and were designated as omental DCs (47). We too observed that 4–5% of OFB walkout cells coexpress CD45 and CD11c, but we also found that most of these cells (80%) coexpressed CD19 and appear to be nonstimulatory in functional assays (see Fig. 6, A and C).
estimate of functional CD11c<sup>high</sup>MHC-II<sup>high</sup>CD19/B220<sup>−</sup> DC frequency in OFB walkout suspensions is therefore ~1%. Generally, previous investigations have not yielded phenotypic evidence of DCs in resting murine omentum (5, 7–9). Omental dendritic F4/80<sup>−</sup> macrophages expressing high levels of class II MHC have been described (9), but their functionality as APCs has not been explored. We readily detected F4/80<sup>−</sup> cells in OFB walkout cells but these were CD11c<sup>−</sup> and nonstimulatory (data not shown).

Cross-presentation activity, where exogenous Ag is processed and accesses class I MHC peptide loading compartments, is thought to reflect a functional specialization among DCs enabling them to reflect more effective immune surveillance functions or possibly self-tolerance-inducing functions. Parallel phenotypic and functional analyses revealed that OFB DCs could cross-present exogenous Ag to naive class I MHC-restricted T cells. Cross-presentation function of OFB DCs was demonstrated with exogenous soluble OVA and spleen cells pulsed with OVA, with the latter being considered a relatively rigorous test of cross-presentation activity (32, 33). After i.p. injection of E. coli, OFB DCs also acquired, processed, and cross-presented bacteria-expressed OVA to OT-I CD8<sup>+</sup> T cells, demonstrating that the OFB can, in principle, perform at least some sentinel function within the peritoneal cavity. Whether OFB DCs that acquire bacterial Ag persist and present Ag in the OFB or migrate elsewhere to present Ag in secondary lymphoid organs is currently unresolved.

With some exceptions (Refs. 53, 54 and references therein), DCs that reside in secondary lymphoid organs and cross-present Ag are CD8<sup>−</sup>CD11b<sup>−</sup> (31, 32). In contrast, cross-presentation OFB DCs exhibit a distinct CD8<sup>+</sup>CD11b<sup>high</sup> status of OFB DCs marks them as members of the visceral fat depots and thereby extend a framework for how this crosspresenting function (56, 57).

References