Subepithelial Myofibroblasts are Novel Nonprofessional APCs in the Human Colonic Mucosa

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The human gastrointestinal mucosa is exposed to a diverse normal microflora and dietary Ags and is a common site of entry for pathogens. The mucosal immune system must respond to these diverse signals with either the initiation of immunity or tolerance. APCs are important accessory cells that modulate T cell responses which initiate and maintain adaptive immunity. The ability of APCs to communicate with CD4+ T cells is largely dependent on the expression of class II MHC molecules by the APCs. Using immunohistochemistry, confocal microscopy, and flow cytometry, we demonstrate that α-smooth muscle actin+ CD90+ subepithelial myofibroblasts (stromal cells) constitutively express class II MHC molecules in normal colonic mucosa and that they are distinct from professional APCs such as macrophages and dendritic cells. Primary isolates of human colonic myofibroblasts (CMFs) cultured in vitro were able to stimulate allogeneic CD4+ T cell proliferation. This process was dependent on class II MHC and CD80/86 costimulatory molecule expression by the myofibroblasts. We also demonstrate that CMFs, engineered to express a specific DR4 allele, can process and present human serum albumin to a human serum albumin-specific and DR4 allele-restricted T cell hybridoma. These studies characterize a novel cell phenotype which, due to its strategic location and class II MHC expression, may be involved in capture of Ags that cross the epithelial barrier and present them to lamina propria CD4+ T cells. Thus, human CMFs may be important in regulating local immunity in the colon. The Journal of Immunology, 2006, 177: 5968–5979.

The intestinal mucosal immune response in healthy individuals is characterized by a balance between immunity, which protects mucosal surfaces from harmful microbes, and tolerance, which permits the intestinal mucosa to interact in a nonpathologic way with the commensal bacteria and dietary Ags to which it is constantly exposed. Although T cells are central to an effective adaptive response, much of their function is highly orchestrated by their interactions with MHC class II-expressing APCs. Activation of T cells requires their recognition of specific Ags bound by MHC proteins on APCs, which in the case of CD4+ T cells, are class II MHC heterodimers. TCR recognition of MHC-Ag complexes alone is insufficient for optimal T cell activation. The APC must also express important costimulatory molecules that interact with receptors on T cells and thus regulate them. The best characterized costimulatory molecules are B7.1 (CD80) and B7.2 (CD86), which engage the coreceptors CD28 and CTLA-4 on CD4+ T cells (1). The interaction of B7 with CD28 enhances T lymphocyte activation (2, 3) and Ab-mediated interference of the B7-CD28 interaction blocks this activation (3), confirming the essential role for B7 as a costimulator. In fact, Ag presentation in the absence of B7 costimulation has been shown to lead to T cell anergy (4). Low levels of B7 expression on APCs limits the response of activated T cells due to preferential signaling through the inhibitory receptor CTLA-4 (1, 5, 6). The importance of these costimulatory molecules in mucosal defenses was confirmed by studies where B7-1 and B7-2 knockout mice have reduced mucosal and systemic anti-Salmonella Ab responses (7).

Professional APCs residing in the intestinal mucosa include B lymphocytes, macrophages, and dendritic cells (DCs). These APCs play important roles in the regulation of oral tolerance and the induction of immune responses to specific Ags via processes thought to occur mostly in gut-associated lymphoid tissue and possibly in the lamina propria. During inflammatory processes, other cell types have been noted to express class II MHC molecules and have been termed nonprofessional APCs due to their potential to modulate CD4+ T cell responses. Colonic epithelial cells are one example of such cells that express MHC class II molecules, but do so only under conditions of intestinal inflammation, being devoid of class II MHC in healthy individuals (8–11). Except for endothelial cells, MHC class II molecules have not been documented or thoroughly studied on other resident cell types within the colonic lamina propria.

Stromal fibroblasts and myofibroblasts have been implicated in the regulation of mucosal inflammation (12–14). Through their synthesis of cytokines, chemokines, eicosanoids, and extracellular
matrix components, stromal cells affect the recruitment, retention, and activation of immune cells (14–23). The importance of stromal fibroblasts and myofibroblasts to the perpetuation of chronic inflammation has been previously appreciated in other chronic inflammatory diseases such as rheumatoid arthritis (24–27) and chronic airway inflammation (28–34).

The intestinal myofibroblast is a mesenchymal (stromal) cell that expresses features of fibroblast and smooth muscle differentiation. Myofibroblasts express α-smooth muscle actin (α-SMA), yet are negative for other smooth muscle markers such as smoothelin and desmin (16, 17). These cells are also positive for the marker CD90, which in humans represents a useful fibroblast/myofibroblast marker because, in humans, it is not expressed by T lymphocytes (35). The subepithelial, pericryptal fibroblastic sheath of the small intestine and colon is composed of intestinal myofibroblasts. These subepithelial myofibroblasts connect with interstitial fibroblasts to form a network of interconnected mononuclear cells throughout the lamina propria (36). Given their strategic location at the interface between the epithelium and lymphocytes of the lamina propria, intestinal myofibroblasts may be among the first cells to interact with Ags that cross the epithelial barrier and present them to mucosal T lymphocytes, thus playing a pivotal role in mucosal immune homeostasis. Although professional APCs are present in the intestinal mucosa, subepithelial myofibroblasts are more numerous. To understand the unique immune regulation in the intestinal mucosa, all potential APCs should be considered.

In the present study, we demonstrate that human colonic myofibroblasts (CMFs) express MHC class II molecules in situ and when cultured with low concentrations of IFN-γ in vitro. We found that human CMFs express low but significant levels of B7 costimulators and can induce allogeneic CD4+ T cell activation in an MHC class II- and B7-dependent manner. We also show that cultured human CMFs can process and present Ag to Ag-specific T cells. Our data suggests that CMFs may represent a previously unappreciated APC phenotype that may influence immune responses in the colonic mucosa and, therefore, may have an important function in the maintenance of mucosal tolerance and/or immunity.

Materials and Methods

Abs and cytokines

The fluorochrome-conjugated and -unconjugated anti-human α-SMA (clone 1AA) murine mAbs were purchased from Sigma-Aldrich. Murine mAbs to human desmin (clone D33), CD68 (clone KP1), human epithelial Ag EP4 (clone Ber-EP4), MHC class II α-chain (clone TAL1B5), and MHC class II β-chain (clone CR3/43) were all obtained from DakoCyto- mation. Murine mAbs against human HLA-DR (clone L243) and human HLA-DP (clone Hu2F11), and -DR (clone Hu2F10) were purchased from R&D Systems. Fluorochrome-conjugated forms of IgG1, IgG2a, IgG2b, and IgG3 isotype controls and mAbs directed against human CD11c (clone B-ly6), CD19 (clone HIB19), CD80 (clone L307.4), CD86 (clone 2313(FUN-1)), and CD90 (clone 5E10) were purchased from BD Biosciences. Fluorochrome-conjugated Abs against human CD45RA (clone HI100), CD45RO (clone UCHL1), as well as murine IgG1, and IgG2a isotypes (functional grade) were purchased from eBioscience. Anti-HLA-DR rat mAbs (clone YD1/63.4.10) were purchased from Abcam. Alexa Fluor (AF) 488-labeled donkey anti-mouse IgG (H+L), AF 594-labeled rabbit anti-rat IgG (H+L) and Zenon Mouse IgG labeling kits were purchased from Molecular Probes. Fluorochrome-conjugated goat anti-mouse IgG (H+L) Abs were purchased from Caltag Laboratories. The hybridomas secreting anti-human MHC class II mAbs (clones IVA12 and L243) were from the American Type Culture Collection, and were used to produce the corresponding Abs. Recombinant human IFN-γ, CTLA-4-Fc, and IgG1Fc chimera (functional grade) were obtained from R&D Systems.

Human colonic tissue specimens

For immunohistochemical studies and CMF isolation, full thickness fresh human tissue samples were obtained from discarded surgical resection material of colon in compliance with protocols approved by the University of Texas Medical Branch (UTMB) Institutional Review Board. Areas of uninvolved colon tissue from patients undergoing colectomy for colon cancer were studied. Archival tissue samples were also used for immunostaining as described below.

Primary CMF cultures

Normal colonic 18Co myofibroblast primary isolate was obtained from the American Tissue Culture Collection (ATCC no. CRL 1459, passages 11–15). 18Co was used in the study as a representative CMF. Additional primary cultures of subepithelial CMFs were generated according to the method described by Mahida et al. (37, 38). The purity of isolated cells was analyzed by immunohistochemistry and flow cytometry analysis as described below. Studies were performed with primary CMFs at passages 5–9. Cells were cultured at 37°C in 5% CO2 atmosphere in complete MEM, which contain MEM base supplemented with nonessential amino acids, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin/streptomycin (Gibco), and 10% heat-inactivated FBS (Sigma-Aldrich).

Isolation of peripheral blood CD4+ T cells

PBMC were prepared from the blood of healthy donors by density gradient centrifugation over Ficoll-Paque Plus (Amsahem Biosciences) according to the manufacturer’s instructions. Human resting CD4+ T cells were then purified from these PBMC by negative selection using a commercially available CD4+ T cell isolation magnetic bead kit II (Miltenyi Biotec) according to the manufacturer’s instructions. Negative selection was chosen to avoid accidental activation of CD4+ T cells during purification. The purity of isolated CD4+ T cells (>97%) was confirmed by flow cytometry.

Human lamina propria mononuclear cell and acute myofibroblast/fibroblast preparations

Discarded excess human colonic surgical tissue was acquired and the mucosal propria removed by dissection. Colonic lamina propria mononuclear cells were isolated by a modification (omission of Dispase) of a protocol provided by Dr. R. Edwards (University of California, Irvine, CA) (39). Briefly, mucosal fragments were minced, washed three times in calcium- and magnesium-free HBSS (In vitroLife Technologies). Rinsed tissue fragments were resuspended in HBSS containing 5 mM EDTA and incubated for 15 min at 37°C with orbital rotation (250 rpm). Fragments were then washed five times in HBSS containing 1 mM DTT (Sigma-Aldrich) and 1 mM EDTA (Sigma-Aldrich) for 15 min at 37°C with orbital rotation. This was done to remove epithelial cells. After the last incubation the tissue fragments were washed two times with HBSS, placed in HBSS-based enzymatic solution containing 2000 U of collagenase I (Sigma-Aldrich) and incubated for 3 h at 37°C with agitation at 250 rpm. Cell suspensions were passed through a Falcon cell strainer (70-μm mesh) (BD Biosciences), washed twice with PBS containing 2 mM EDTA and 0.5% FBS. The resulting single-cell suspension was then processed for immunostaining followed by FACS as described below.

The myofibroblast/fibroblast cell population was isolated from the lamina propria mononuclear cells by positive selection using anti-fibroblast microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. Purity of the isolated myofibroblast/fibroblast population (>98%) was assessed by immunostaining with fluorochrome-conjugated anti-CD90 and α-SMA mAbs followed by FACS analysis.

Generation of retroviral infectants

The HLA-DRα and -DRβ containing retroviral vectors have been described and were provided by Dr. R. Hershberg (Corixa, Seattle, WA) (9). Briefly, the HLA-DRα and -DRB1*0401 cDNA were expressed under the control of the CMV-IE promoter and the cDNA for the dominant selectable marker (hygromycin resistance for the DRA vector and neomycin resistance for the DRB1*0401 vector) was expressed under the control of the viral long terminal repeat. Cells were first infected with the DRA vector, selected as pools, and then infected with the DRB1*0401 vector. Pools of hygromycin- and neomycin-resistant, infected 18Co cells were expanded and evaluated for HLA-DR expression by flow cytometry analysis, as described below. In addition, the constitutive 18Co HLA haplotype was determined by the Tissue Typing Laboratory at UTMB under the direction of Dr. S. Vaidya (UTMB, Galveston, TX) before use of these cells for the HLA-DR transfection (18Co have the DR1/DR12*, D65+ haplotype).

Immunohistochemistry

Immunohistochemical staining of archival human specimens was performed using an established avidin-biotin detection method (Vector Laboratories) as described previously (36). Briefly, 4- to 10-μm-thick sections.
of the paraffin-embedded tissue blocks were cut, mounted on positively charged glass slides, and dried in an oven at 56°C for 30 min. The sections were deparaffinized in xylene and then rehydrated in graded ethanol and water. Endogenous peroxidase was blocked by incubation in 3% methanol peroxide for 10 min. Ag retrieval was accomplished by pretreatment of the sections with citrate buffer at pH 6.0 for 20 min at 56°C in a microwave oven and then allowing the sections to cool to 20°C at room temperature. Nonspecific endogenous protein binding was blocked using the avidin-biotin blocking kit (Vector Laboratories). The sections were then incubated with biotin-labeled primary Ab solution at room temperature for 30 min. The sections were subsequently subjected to the following sequential treatments: staining with streptavidin-biotin peroxidase detection kit (DakoCytomation), reaction with 3,3'-diaminobenzidine, and counterstaining with hematoxylin.

**Confocal microscopy**

Unfixed frozen tissue sections of colonic mucosa, obtained as described above, were blocked with normal mouse and goat serum (1/100 in PBS) for 30 min at room temperature. Samples were then incubated with HLA-DR (1/400) rat mAbs, or rat IgG2a isotype control (same dilution) for 30 min at room temperature. Samples were then stained with AF 592-conjugated rabbit anti-rat IgG Abs (1/400) for 30 min. During the next step, samples were stained with anti-α-SMA mAbs (dilution 1/200 in PBS) or mouse normal IgG2a isotype control at room temperature for the same time period. Samples were probed with donkey anti-mouse IgG (H+L) Abs conjugated with AF 488. Each staining step was followed by three washes with PBS. Acetone-fixed frozen tissue sections were stained according to similar protocols. For this staining, AF 488-conjugated anti-α-SMA mAbs (dilution 1/200 in PBS), AF 568-conjugated anti-human MHC class II mouse mAbs (dilution 1/300), AF 647-conjugated murine mAbs against human epithelial Ag (dilution 1/400) or appropriated mouse IgG isotype controls were used. Confocal microscopy was performed with a Zeiss LSM510 META laser scanning confocal microscope (Carl Zeiss). AF 488 staining was observed using an excitation wavelength of 488 nm and an emission wavelength of 505–530 nm (argon/ion laser). The AF 568 and AF 594 staining were visualized with an excitation wavelength of 543 nm and an emission wavelength at 585–615 nm (green helium/neon laser). The AF 647 staining was detected with an excitation wavelength of 633 nm and an emission wavelength longer than 650 nm (red helium/neon laser).

**Flow cytometry**

CMFs were detached from the culture flasks with treatment with 0.04% EDTA at 37°C for 15 min, followed by two washes with cold PBS. CMFs, T cells, or human colonic mucosal cells were stained for surface and/or intracellular markers according to each experimental need. Single-, double-, or triple-color fluorochrome-conjugation was performed according to standard and intracellular FACS staining protocols described by BD Pharmingen. Briefly, aliquots of 1×10^6 cells were resuspended in FACS buffer (PBS containing 1% BSA and 0.1% NaN3). The cells were initially incubated in FACS buffer containing 10% human AB serum (ICN Biomedicals) for 20 min at 4°C. Cells were then washed two times with cold PBS and incubated with conjugated or unconjugated specific Abs (1μg/ml) for 30 min, and then incubated with fluorescein-conjugated secondary Ab if necessary) or other color fluorochrome-conjugated Abs (for multiple-color FACS analysis) for an additional 30 min. Cells were washed three times with PBS before each step of immunostaining. Cells were then fixed using 500μl of 1% paraformaldehyde in PBS and analyzed in a FACScan cytometer (BD Biosciences). The detection of intracellular markers (such as α-SMA and desmin) was performed by fixing 1×10^6 cells with BD Cytofix/Cytoperm solution (BD Pharmingen) for 20 min at 4°C, followed by three washes with Perm/Wash buffer (BD Pharmingen), and incubation with fluorescein-conjugated- or unconjugated appropriate Abs for 45 min, followed by staining with secondary fluorescein-conjugated Abs, when indicated. Cells were washed three times with Perm/Wash buffer before each step of intracellular immunostaining and analyzed in FACSscan and FACSCan cytometers. The FACS analysis was conducted with op-time, differential interference, and fluorescein excitation. Single-color FACS analysis was performed by gating on the typical forward and side light scatter characteristics of analyzed cells, and histograms were plotted for each Ab to determine the mean fluorescence intensity. Nonspecific staining was determined for each Ab using isotype controls. Samples were considered negative if the percentage of positive cells was 2% or less.

Three-color FACS analysis was performed for analysis of CMF populations in colonic lamina propia mononuclear cell suspensions. Percentage of cells that were Ab positive was calculated by comparison with the corresponding isotype control. A total of at least 10,000 events was scanned for each experimental condition. Flow cytometry data were analyzed with WinMDI 2.8 software (The Scripps Research Institute).

**Western blot analysis**

Western blot analysis was performed as previously described (40). Briefly, cells were washed with ice-cold PBS and lysed in Laemmli sample buffer (10% glycerol, 5% 2-ME, 2% SDS, 0.002% bromphenol blue, and 62.5 mM Tris-HCl (pH 6.8). Ten micrograms of protein was fractionated in 10% SDS-polycrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). The membranes were saturated with 5% fat-free dry milk in TBS (50 mM Tris, pH 7.5, 150 mM NaCl) with 0.05% Tween 20 (TBST) for 2 h at room temperature. Blots were then incubated overnight with the appropriate primary Ab, diluted in 5% BSA, TBST. After washing with TBST solution, blots were further incubated for 1 h at room temperature with the appropriate peroxidase-conjugated secondary Ab for chemiluminescence detection. Blots were then washed three times in TBST before visualization. Chemiluminescent detection was performed using the Enhanced Chemiluminescence Detection kit (Amersham Biosciences) according to the supplier’s recommendations.

**Measurement of IL-2 production**

Acutely isolated myofibroblast/fibroblasts or CMF primary cultures (5×10^6 cells/well), purified resting CD4+ T cells (2×10^6 cell/well) or CMF:CD4+ T cell cocultures were incubated in complete MEM. CMF cultures were stimulated with IFN-γ (100 U/ml) for 7 days in complete medium prior use in coculture experiments. Each culture condition was performed in triplicate in 96-well flat-bottom microtiter plates in 0.2 ml of medium/well. Cells were incubated for 48 h at 37°C in 5% CO2. IL-2 produced in cell culture supernatants was determined using a human IL-2 ELISA kit (BD Pharmingen) according to the manufacturer’s instruction. To detect IL-2 production by the 17.9 T cell hybridoma, described below, we used a mouse IL-2 ELISA kit (BD Pharmingen). Results were expressed in pg/ml as the mean ± SE of triplicate wells.

**Allogeneic T cell proliferation assay**

CMF primary cultures were stimulated with IFN-γ (100 U/ml) for 7 days before study. Irradiated CMF (5×10^6 cells/well) or CMF:CD4+ T cell cocultures were incubated in complete MEM. CMF cultures were stimulated with IFN-γ (100 U/ml) for 7 days in complete medium prior use in coculture experiments. Each culture condition was performed in triplicate in 96-well flat-bottom microtiter plates in 0.2 ml of medium/well. Cocultures were incubated for 4 days at 37°C in 5% CO2. A total of 1μCi of [3H]thymidine was added 18 h before cell harvesting. At the end of the cultures, cells were harvested onto glass fiber filter mats and [3H]thymidine incorporation (cpm) was determined using a liquid scintillation counter (PerkinElmer). Results were expressed in cpm as the mean cpm ± SE of triplicate cultures. The stimulation index (SI) was also calculated and represents the ratio between the proliferative response (cpm) of CD4+ T cells in the presence of CMFs and T cells alone.

**Ag presentation assays**

Human serum albumin (HSA)-specific and HLA-DRB1*0401-restricted T cell hybridoma 17.9 was provided by Dr. R. Hershberg. For the Ag presentation assays using the HLA-DRB1*0401-transfected and nontransfected CMF 18Co cells, cells were grown at confluency in T75 flasks in MEM medium. Cells were trypsinized and plated at 5×10^5 cells/well in a flat-bottom 96-well plate and allowed to adhere overnight. The cells were then pulsed with HSA or tetanus toxoid (TT) Ag (10 μg/ml, negative control) overnight and then Ag not internalized by the cells was removed. A total of 10^5 hybridoma cells was then added and cocultured with CMFs for 48 h, after which cell culture supernatants were collected and IL-2 production was measured as described above.

**Statistical analysis**

Unless otherwise indicated, the results are expressed as the mean ± SE of data obtained from at least three independent experiments done with triplicate sets in each experiment. Differences between means were evaluated by ANOVA using Student’s t test for multiple comparisons. Values of p<0.05 were considered statistically significant.
Results

HLA-DR expression by CMFs in normal human colonic mucosa

Because subepithelial myofibroblasts represent a cell population that is strategically situated to interact with Ags that cross the epithelial barrier and T lymphocytes in the lamina propria, it was important to establish the relationship of this cell to all other cell types capable of presenting Ags to T lymphocytes. We performed immunohistochemical studies of myofibroblast distribution in normal human colonic mucosa. CMFs were identified in colonic mucosa on the basis of their morphology, subepithelial location, positive immunoreactivity for α-SMA, and negative immunoreactivity for desmin (smooth muscle marker), as previously reported (17, 36) (Fig. 1, A–C). We did not observe colocalization between α-SMA and CD68 (macrophage marker) in studies of immunohistochemical step sections (Fig. 1D), nor between α-SMA and CD11c (DC marker) or CD83 (activated DC marker) (Fig. 1, E and F). These data indicate that CMFs in normal colon represent a cell population that is phenotypically distinct from macrophages and DCs. As reported by Silva et al. (41), CD11c

CD83

DCs represent only a small fraction of lamina propria cells in normal colonic crypt. A Subepithelial pericryptal CMFs (in green) were identified using anti-human α-SMA murine mAbs (clone 1A4) and visualized with AF 488-labeled donkey anti-mouse IgG (H+L). B, HLA-DR staining (in red) of colonic mucosa was performed by using anti-HLA-DR rat mAbs (clone YD1/63.34.10) and visualized with AF 594-labeled rabbit anti-rat IgG (H+L). C, Colocalization (in orange and yellow) of α-SMA−CMFs and HLA-DR highlighted with arrows. D–G, High power resolution confocal image analysis of triple immunofluorescent staining of an acetone-fixed cryosection (longitudinal section) of a normal human colonic crypt. D, Subepithelial pericryptal CMFs (in green) were identified using the anti-human α-SMA mAbs. E, MHC class II staining (in red) was realized by using anti-MHC class II mouse mAbs (clone IVA12) conjugated with AF 568. F, Colonic epithelium (in blue) was detected by using murine mAbs against HEA (clone Ber-EP4) conjugated with AF 647. G, Merged images D–F clearly demonstrate coexpression (orange-yellow staining) of MHC class II and α-SMA by CMFs (highlighted with arrows) that is separate from minimal colocalization of MHC class II and HEA in the epithelial cells in the normal colonic mucosa. Confocal microscopy was performed by using a Zeiss LSM510 META laser scanning confocal microscope. Calibration bars are 20 μm.

FIGURE 1. CMFs are located subjacent to the basement membrane. Immunohistochemical analysis of archival human colon sections. Specific Ag staining is shown in brown. Arrows highlight the subepithelial network of interconnected myofibroblasts just below the epithelial basement membrane. Longitudinal sections of normal human colonic crypts stained for (A) α-SMA and (B) desmin. C–G, Step cross-section of normal human colonic crypts stained for (C) α-SMA, (D) CD68, (E) CD11c, (F) CD83, and (G) HLA-DR. Original magnification, ×400.
colonic mucosa. Furthermore, macrophages in the human colonic mucosa are concentrated in the subepithelial region under the surface epithelium (42–44) and are relatively sparse along the colonic crypts.

Step sections of colonic crypts from normal tissue were also analyzed for HLA-DR expression. HLA-DR \( ^{1}/H11001 \) cells were found subjacent to the crypt epithelium (Fig. 1G), corresponding to the location of \( \alpha\text{-SMA}^{+} \) pericryptal CMFs (Fig. 1C). Additional HLA-DR staining in the lamina propria corresponded to areas occupied by the less frequent macrophages and DCs. The expression of HLA-DR by CMFs in normal human colonic mucosa was confirmed by confocal microscopy using double immunostaining for HLA-DR and \( \alpha\text{-SMA} \) (red, Fig. 2B) staining in cells consistent with a pericryptal CMF phenotype (\( \alpha\text{-SMA}^{+} \) subepithelial cells, in green, Fig. 2A). The orange-yellow staining in Fig. 2C (white arrows), resulting from merged images, indicates \( \alpha\text{-SMA} \) and HLA-DR coexpression by pericryptal CMFs in normal human colonic mucosa. Furthermore, we performed triple immunostaining for MHC class II, \( \alpha\text{-SMA} \), and human epithelial Ag (HEA) to differentiate MHC class II expression by CMFs, from MHC class II expression on basolateral membranes of epithelial cells (Fig. 2, D–G). High power resolution confocal image analysis demonstrated that MHC class II in the normal colon was expressed by \( \alpha\text{-SMA}^{+} \) lamina propria cells (e.g., CMFs), although occasional faint MHC class II basolateral staining of epithelial cells was also observed as previously reported (10). Thus, this data demonstrates that normal CMFs represent a distinct cell population that constitutively express class II MHC molecules.

**Immunophenotypic characterization of CMFs in normal colonic lamina propria mononuclear cell preparations**

To confirm the observations by immunohistochemistry using an independent assay where the phenotypic characterization of the resident cells could be determined individually, single-cell suspensions of lamina propria mononuclear cells were generated from human colonic mucosa specimens as described in *Materials and Methods*. These cell suspensions were subsequently analyzed by multicolor flow cytometry analysis (Fig. 3). In addition to lymphocytes, macrophages, monocytes, and other leukocytes, these preparations also contain additional resident cell types such as myofibroblasts, fibroblasts, endothelial cells, and muscularis mucosa-derived smooth muscle cells. In these preparations, CMFs are identified as \( \alpha\text{-SMA}^{+} \) (myofibroblast/muscularis mucosa cell marker) positive, and CD90 (e.g., Thy-1, fibroblast/myofibroblast marker) positive cells. Myofibroblasts (\( \alpha\text{-SMA}^{+}\text{CD90}^{-} \)) comprised 12.8 \( \pm \) 3.23%, whereas fibroblasts (\( \alpha\text{-SMA}^{+}\text{CD90}^{+} \)) and muscularis mucosa cells (\( \alpha\text{-SMA}^{+}\text{CD90}^{-} \)) represented 13.2 \( \pm \) 2.03% and 7.0 \( \pm \) 3.91%, respectively, of the total lamina

**FIGURE 3.** CMFs in lamina propria mononuclear cell preparations express HLA-DR and B7 costimulatory molecules. Lamina propria mononuclear cells, prepared as described in *Materials and Methods*, were subjected to TriColor immunostaining followed by flow cytometry analysis as described in *Materials and Methods*. A, Dot plot of total fresh colonic mucosal cell preparations, where 88.36 \( \pm \) 5.25% of total events were gated in R1. B, Cells bearing the myofibroblast phenotype (\( \alpha\text{-SMA}^{+}\text{CD90}^{-} \), R2) represent 12.82 \( \pm \) 3.23% of the gated colonic mucosal cells. C, Expression of HLA-DR, CD80, and CD86 by \( \alpha\text{-SMA}^{+}\text{CD90}^{-} \) cells (gated in R2). Results are calculated as the mean value of five experiments \( \pm \) SD. A representative experiment is shown (\( n = 5 \)).
Table I. Expression of surface Ags and receptors by acutely isolated CMFs

<table>
<thead>
<tr>
<th>Markers</th>
<th>CMFs</th>
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<tr>
<td>CD11c</td>
<td>0.58 ± 0.29</td>
</tr>
<tr>
<td>CD19</td>
<td>0.46 ± 0.31</td>
</tr>
<tr>
<td>CD68</td>
<td>1.59 ± 0.57</td>
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<tr>
<td>EF-4</td>
<td>1.62 ± 0.93</td>
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<tr>
<td>HLA-DR</td>
<td>87.81 ± 5.46</td>
</tr>
<tr>
<td>CD80</td>
<td>42.15 ± 12.27</td>
</tr>
<tr>
<td>CD86</td>
<td>65.14 ± 15.20</td>
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* Percentage of cells expressing cell surface marker (FACS analysis) is shown as mean ± SD from five experiments.

In contrast to what we observed in vivo, cultured CMFs expressed B7 family costimulatory molecules CD80 and CD86. Of all cell types expected to express MHC class II molecules, CMFs express MHC class II molecules in vitro.

To study MHC class II molecule expression and function by CMFs independently of other cell types, we have established primary CMF cultures from normal human tissue. A commercially available human CMF primary isolate, 18Co, was also used as a representative CMF (45). Phenotypic characterization of cultured CMFs and 18Co cells has demonstrated that they are uniformly positive for α-SMA and CD90, but negative for desmin (Fig. 4). Additionally, these CMFs were negative for cytokeratin, epithelial marker EP4, and lymphoid, monocytic, and DC markers (CD45RA, CD45RO, CD19, CD68, and CD11c) (Fig. 4).

In contrast to what we observed in vivo, cultured CMFs expressed low levels of MHC class II molecules; these levels, however, could be readily restored by culture in the presence of low concentrations of IFN-γ. Western blotting demonstrated faint expression of HLA-DRα and HLA-DRβ chains of appropriate molecular mass in unstimulated cells that became readily detectable after treatment with IFN-γ (100 U/ml) (Fig. 5A). As little as 1.0 U/ml IFN-γ elevated MHC class II expression in cultured CMFs (Fig. 5B). MHC class II expression on the surface of cultured CMFs was confirmed by flow cytometric analysis of at least four independent isolates (Fig. 5C).

Primary CMF cultures induce allogeneic proliferation of resting CD4+ T cells

We next explored the functional consequences of MHC class II expression by human CMFs. Allogeneic CD4+ T cell proliferation assays were performed to test the ability of CMFs to activate resting CD4+ T cells in an MHC class II-dependent fashion. Coculture of irradiated class II MHC-expressing 18Co cells with CD4+ T cells caused a 28.5 ± 3.96-fold increase in CD4+ T cell proliferation (n = 5) and increased IL-2 levels (852 ± 125.6 pg/ml, p < 0.05) in coculture supernatants (Fig. 6). Both proliferation and IL-2 production were inhibited (>90%) when anti-class II MHC Abs (anti- HLA-DR, -DQ, and -DP mixture, clones L243 and IVA12) were included (Figs. 6). In the blocking experiments, isotype control Abs did not alter the proliferative or IL-2 responses, proving the specificity of the MHC class II-blocking mixture (Figs. 6). Similar results to those shown in Fig. 6 were obtained with two other cultured isolates, CMF3 and CMF5 (data not shown). Moreover, by using a standard MLR, we compared the ability of CMFs to activate resting CD4+ T cells with that of conventional APCs. The allogeneic proliferation of T cells induced by 18Co was comparable to that induced by irradiated human PBMC (data not shown). Taken together, these data indicate that CMFs can activate CD4+ T cells in an MHC class II-dependent manner.

CMF-induced allogeneic CD4+ T cell proliferation depends on B7 family costimulatory molecules

As with class II MHC expression, CD80 (B7-1) and CD86 (B7-2) expression in cultured CMFs cultured was not readily detected as it was in freshly isolated CMFs. Detectable expression of the costimulators was not restored by IFN-γ treatment alone. However, when 18Co cells were cocultured with CD4+ T cells for 24–72 h, low but significant surface expression of CD80 and CD86 was observed by CMFs (α-SMA+/CD90+ cells) (Fig. 7A). We then assessed the functional activity of B7 molecules expressed by CMFs upon allogeneic proliferation of CD4+ T cells by including a CTLA-4-Fc soluble human fusion protein in the culture medium. CD80 and CD86 bind to CD28 and CTLA-4 on T cells and CTLA-4 is a high-affinity receptor for both CD80 and CD86. The soluble fusion protein acts as an antagonist that prevents the binding of CD28 to either CD80 or CD86. As presented in Fig. 7, B and C, allogeneic proliferation and IL-2 production were suppressed by 84 and 89%, respectively, in the presence of the soluble CTLA-4-Fc fusion protein (1 μg/ml). Human IgG at the same concentration did not alter 18Co induced allogeneic CD4+ T cell proliferation or IL-2 production. These results suggest that CD80 and CD86 are functionally involved in CMF-induced allogeneic CD4+ T cell activation.

Acutely isolated myofibroblasts/fibroblasts induce allogeneic proliferation of CD4+ T cells in MHC class II- and B7-dependent manner

To clarify whether the ability of cultured, IFN-γ-treated myofibroblasts to activate allogeneic resting CD4+ T cells in an MHC class II- and B7-dependent fashion represents a property that reflects the capabilities of these APCs in vivo, it is importantly...
show that acutely isolated, non-IFN-γ-treated myofibroblasts/fibroblasts have a similar capability. The myofibroblast/fibroblast population from normal colonic tissue was purified from acute lamina propria mononuclear cells preparations by positive selection using antifibroblast microbeads. Coculture of class II MHC-expressing myofibroblasts/fibroblasts with CD4+ T cells induced significant increase in IL-2 production ($420 \pm 162.6$ pg/ml, $p < 0.05, n = 3$) in coculture supernatants (Fig. 8). This IL-2 production was abrogated when anti-class II MHC Abs or soluble CTLA-4-Fc fusion protein were added to the coculture. In the blocking experiments, neither isotype control Abs nor human IgG-Fc altered the IL-2 response by allogeneic CD4+ T cells in cocultures, proving the specificity of the MHC class II and B7-molecule blocking. These results with acutely isolated, non-IFN-γ-treated myofibroblasts suggest that MHC class II and B7 molecules expressed by CMFs in vivo are functionally active. Moreover, these results suggest that CMFs may be involved in Ag presentation processes in the normal colon.

**CMFs can process and present Ags to CD4+ T cells in an MHC class II-restricted manner**

Although the allogeneic stimulation of CD4+ T cells by CMFs provided confirmation of the functional expression of both class II MHC and costimulatory molecules on their surface, additional hallmarks of APCs include their ability to internalize, process, and present markers for other cell types: i.e., smooth muscle cells (desmin), epithelial cells (EP4), naïve and memory T cells (CD45 RA and CD45 RO), B cells (CD19), monocytes (CD68), and DC (CD11c). Appropriate Ab isotype control for each (filled histogram) was included and superimposed (open histogram). One of four representative experiments is shown for 18Co. Similar results were obtained with the other primary isolates (CMF3, CMF5, CMF6) used in these studies.

**Discussion**

Intestinal myofibroblasts, by virtue of their location at the interface between the epithelium and the lamina propria, may play pivotal roles in mucosal homeostasis by modulating information transfer between these tissue compartments (16, 17, 46). In the present work, we have shown that in the noninflamed human large intestine, CMFs represent a significant fraction of resident lamina propria cells that express MHC class II molecules. We have also shown that cultured CMFs induce allogeneic proliferation of human CD4+ T cells in a process dependent upon MHC class II and B7 family costimulatory molecule expression by CMFs. Further, we have demonstrated that cultured CMFs can process and present Ags to CD4+ T cells in an MHC class II-restricted manner. Our data suggest that CMFs represent a previously unappreciated nonprofessional APC type capable of modulating mucosal immune responses.

CMFs are phenotypically distinct from other professional and nonprofessional APCs of the intestinal mucosa. Immunohistochemical analyses showed no colocalization between α-SMA, a marker for CMFs, and markers of known professional or nonprofessional APCs such as monocytes/macrophages, DCs, B lymphocytes, or epithelial cells. FACs analysis of colonic mucosal cell preparations confirmed our immunohistochemistry data: CMFs did...
not express markers of known professional APCs. The use of double immunostaining for α-SMA and CD90 (i.e., Thy-1) allowed us to distinguish CMFs from muscularis mucosa cells (α-SMA+CD90−) and fibroblasts (α-SMA−CD90+) during FACS analysis. CD90 is a well-known surface adhesion molecule that has been investigated over three decades. It is familiar to many investigators as a murine T cell marker. In contrast to rodents, CD90 is not expressed by human T cells and is only expressed by fibroblasts, myofibroblasts, neuronal cells, blood stem cells, and activated microvascular endothelial cells (35, 47–49). In the normal human intestinal mucosa, CD90 expression is mostly associated with myofibroblasts and fibroblasts (35, 47–49). In contrast to myofibroblasts, freshly isolated fibroblasts do not express α-SMA. Thus, in our FACS analysis of acute colonic mucosal cell preparations, cells bearing double positivity for α-SMA and CD90 were identified as CMFs. Interestingly, we observed heterogeneity in the α-SMA expression by acutely isolated CD90+ myofibroblast population. This heterogeneity may be due to the variation in the local colonic cytokine milieu, because it has been reported that α-SMA expression by myofibroblasts is up-regulated by TGF-β (50) and down-regulated by IFN-γ (51). These cytokines have been reported to be produced in the normal colonic mucosa (52, 53).

We report here that CMFs, which form a network of interconnected mononuclear cells below the epithelial basement membrane, express HLA-DR molecules in the normal, noninflamed colonic mucosa. It has been reported previously that MHC class II...
(HLA-DR, - DP, - DQ)-expressing cells exist in a band-like organization beneath the surface epithelium of human colon (42). Although this is the first report demonstrating that human CMFs express MHC class II in situ, it is not without precedence for other mesenchymal cells. Other fibroblasts and myofibroblasts with this capacity are human dermal fibroblasts (54, 55), human gingival fibroblasts (56), human periodontal ligament fibroblasts (57), synovial fibroblasts (58), human orbital myofibroblasts (59), as well as other members of the myofibroblast family, such as mouse astrocytes (60) and mouse neonatal microglia (61).

In contrast to other cell phenotypes that have been considered as potential APCs in the intestinal mucosa due to their expression of class II MHC molecules during inflammatory conditions, CMFs in situ and freshly isolated CMFs were found to constitutively express MHC class II molecules. This expression was significantly reduced as CMFs were cultured in vitro, but easily induced by incubation with low concentrations of IFN-γ (1 μg/ml). Human IgG-Fc protein (1 μg/ml) was used as a blocking specificity control for the CTLA-4-Fc soluble human fusion protein. Assays shown in B and C were performed as described in the legend to Fig. 6. * p < 0.05.

FIGURE 7. CMF-induced allogeneic CD4⁺ T cell proliferation depends on B7 family costimulatory molecules. A, CMFs express CD80 and CD86 during coculture with resting CD4⁺ T cells, but not during monoculture. 18Co cells were cultured 7 days in the presence of 100 U/ml IFN-γ to enhance MHC class II expression followed by culture in the presence or absence of CD4⁺ T cells. After an additional 72 h, cells were harvested for flow cytometry analysis. Expression of B7 costimulators (open histogram) by α-SMA⁺ CD90⁺ cells (i.e., CMFs) was realized by TriColor flow cytometry analysis. An isotype control (filled histogram) was included for each staining. B, Allogeneic proliferative response and (C) IL-2 production of CD4⁺ T cells induced by the presence of MHC class II⁺ CMFs is abrogated by the presence of CTLA-4-Fc soluble human fusion protein (1 μg/ml). Human IgG-Fc protein (1 μg/ml) was used as a blocking specificity control for the CTLA-4-Fc soluble human fusion protein. Assays shown in B and C were performed as described in the legend to Fig. 6. * p < 0.05.
Acutely isolated myofibroblast/fibroblasts induce allogeneic proliferation of CD4⁺ T cells in MHC class II- and B7-dependent manner. Acutely isolated myofibroblast/fibroblasts (purified from normal colonic tissue) were cocultured with allogeneic resting CD4⁺ T cells (purified from PBMC by negative selection) in 96-well plates at a CMF:CD4⁺ T cell ratio of 1:4. Cocultures were performed in the presence or absence of anti-HLA DR, DQ, DP Ab mix (clone IVA12 and L243, 10 μg/ml of each) or isotype control (mix of murine IgG1, and IgG2a (10 μg/ml of each)), as well CTLA-4-Fc soluble human fusion protein (1 μg/ml) or human IgG-Fc. IL-2 production by CD4⁺ T cells cocultured with MHC class II⁺ CMFs was assessed in 40-h coculture supernatants using a standard IL-2 ELISA kit (BD Pharmingen). Values represent the mean of IL-2 production in culture supernatants expressed as picograms per milliliter ± SE of the triplicates of three independent experiments with independent blood and myofibroblast/fibroblasts donors (n = 3), *, p < 0.05.

**FIGURE 8.** Acutely isolated myofibroblast/fibroblasts induce allogeneic proliferation of CD4⁺ T cells in MHC class II- and B7-dependent manner. Acutely isolated myofibroblast/fibroblasts (purified from normal colonic tissue) were cocultured with allogeneic resting CD4⁺ T cells (purified from PBMC by negative selection) in 96-well plates at a CMF:CD4⁺ T cell ratio of 1:4. Cocultures were performed in the presence or absence of anti-HLA DR, DQ, DP Ab mix (clone IVA12 and L243, 10 μg/ml of each) or isotype control (mix of murine IgG1, and IgG2a (10 μg/ml of each)), as well CTLA-4-Fc soluble human fusion protein (1 μg/ml) or human IgG-Fc. IL-2 production by CD4⁺ T cells cocultured with MHC class II⁺ CMFs was assessed in 40-h coculture supernatants using a standard IL-2 ELISA kit (BD Pharmingen). Values represent the mean of IL-2 production in culture supernatants expressed as picograms per milliliter ± SE of the triplicates of three independent experiments with independent blood and myofibroblast/fibroblasts donors (n = 3), *, p < 0.05.

**FIGURE 9.** CMFs process and present Ags to CD4⁺ T cells in an MHC class II-restricted manner. IL-2 production by the HSA-specific HLA-DRB1*0401-restricted T cell hybridoma 17.9 was analyzed after 48 h of coculture with HLA-DRB1*0401 (DR4 allele)-expressing 18Co cells, generated as described in Materials and Methods. DR4-expressing 18Co cells were pulsed with Ag (HSA or TT, 10 ng/ml) 12 h before incubation. HSA pulsed noninfected 18Co cells pretreated with IFN-γ (100 U/ml, for 7 days) to express DR1 and DR12 were also used in these experiments. IL-2 production was analyzed using a standard IL-2 ELISA kit (BD Pharmingen). Values represent means of IL-2 levels in culture supernatants expressed in picograms per milliliter ± SE of triplicate wells. Each experimental condition was repeated twice. *, p < 0.05.

In conclusion, our observations suggest that CMFs are novel, local nonprofessional APCs that may participate in the modulation of lamina propria CD4⁺ T cell responses. Further studies will provide insight into the role CMFs may play in the maintenance of homeostasis and immunopathogenesis of inflammatory bowel disease, perhaps leading to the development of novel therapeutic strategies.
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Disclosures

The authors have no financial conflict of interest.

References


predict early engraftment of autologous blood stem cells as well as the total CD34+ cell dose. Bone Marrow Transplant. 25: 435–440.


