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Interactions of T Cells with Fibroblast-Like Synoviocytes: Role of the B7 Family Costimulatory Ligand B7-H3

Chinh N. Tran,* Seth G. Thacker,* Deanna M. Louie,* Jennifer Oliver,* Peter T. White,* Judith L. Endres,* Andrew G. Urquhart,† Kevin C. Chung,‡ and David A. Fox2*

Fibroblast-like synoviocytes (FLS) and T cells can activate each other in vitro, and in vivo interactions between these cells may be important in rheumatoid arthritis (RA), yet FLS lack significant expression of CD28 ligands. We sought to identify molecules homologous to CD28 ligands that are strongly expressed by FLS, and documented strong B7-H3 expression on FLS and by fibroblasts of other tissues, which was unaffected by a variety of cytokines. Western blot analysis of FLS lysates showed predominant expression of the larger, four Ig-like domain isoform of B7-H3. Immunohistological sections of RA synovial tissue showed strong staining for B7-H3 on FLS. Cells expressing B7-H3 were distinct from but in close proximity to cells that expressed CD45, CD20, and CD3. Confocal microscopy of FLS and T cell cocultures showed localization of B7-H3 in the region of the T cell-FLS contact point, but distinct from the localization of T cell CD11a/CD18 (LFA-1) and FLS CD54 (ICAM-1). Reduction of B7-H3 expression on FLS by RNA interference affected interactions of FLS with resting T cells or cytokine-activated T cells. Resting T cells showed increased production of TNF-α, IFN-γ, and IL-2, whereas cytokine-activated T cells showed reduced cytokine production relative to control. However, cytokine production by T cells activated through their TCR was not notably altered by knock-down of B7-H3. These observations suggest that B7-H3 may be important for the interactions between FLS and T cells in RA, as well as other diseases, and the outcome of such interactions depends on the activation state of the T cell. The Journal of Immunology, 2008, 180: 2989–2998.

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Abbreviations used in this paper: RA, rheumatoid arthritis; FLS, fibroblast-like synoviocyte; RNAi, RNA interference; OA, osteoarthritis; ICOS-L, ICOS ligand; eGFP, enhanced GFP; Tck, cytokine-activated T cell; TreT, resting T cell.

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cells (17). Although FLS lack expression of the classic costimulatory molecules B7-1 and B7-2, we hypothesized that FLS might express B7-H3 and that B7-H3 might participate in T cell-FLS interactions. 

Materials and Methods

**FLS isolation and culture**

All procedures involving specimens obtained from human subjects were performed under a protocol approved by the University of Michigan Institutional Review Board. FLS were obtained by collagenase (Worthington Biochemical) digestion of human synovial tissue obtained at arthroplasty or synovectomy from RA or osteoarthritis (OA) joints. RA diagnosis was based upon the presence of at least four of the seven criteria developed by the American College of Rheumatology for RA (24). The diagnosis of OA was based upon characteristic clinical and radiographic features, and confirmed by pathological findings at joint surgery. Cells were maintained in CMRL medium (Invitrogen Life Technologies) supplemented with 10% FCS (Atlanta Biologicals), 2 mM glutamine (Cambrex), 50 U/ml penicillin (Cambrex), and 50 

**T cell generation**

T cells were obtained from peripheral blood by negative selection using RosetteSep Human T cell Enrichment Cocktail (StemCell Technologies) and separation on a Ficoll gradient. Briefly, freshly isolated blood from volunteers was incubated with kit Ab mix for 20 min at room temperature. Blood was diluted 1/1 with 2% normal calf serum/PBS before overlay onto room temperature Ficoll, and spun at 1200 relative centrifugal force (RCF) with the brake off. Theuffy coat containing only T cells was recovered and used for subsequent experiments. T cells were maintained in RPMI 1640 (Cambrex) supplemented with 10% FCS (Atlanta Biologicals), 2 mM glutamine (Cambrex), 50 U/ml penicillin (Cambrex), and 50 

**FIGURE 1.** FLS express the B7 family molecule B7-H3. A, FLS were stimulated with IFN-γ for 72 h. FLS were removed from culture wells with EDTA and stained with mouse anti-human B7-H3 Ab (black line histogram), mouse anti-human ICOS-L (dashed line histogram), or control mouse IgG (gray line histogram). B, The panel of Abs was expanded in a separate experiment to include B7-H3 (black line histogram), PD-L2 (dashed line histogram), B7-H1 (gray line histogram), or mouse IgG control (gray-filled histogram). Staining is representative of two experiments.

**FIGURE 2.** Fibroblasts from various tissue sources express B7-H3. RA FLS, OA FLS, lung fibroblasts, and skin fibroblasts were stimulated with TNF-α, IFN-γ, IL-1β, IL-17, IL-4, or IL-10 for 72 h. Fibroblasts were removed from culture wells with EDTA and stained with mouse anti-human B7-H3, CD54, or control Ab. Staining is representative of two experiments.

**FIGURE 3.** Fibroblasts express the 4IgB7-H3. A, Recombinant 4IgB7-H3 and 2IgB7-H3 were separated on SDS-PAGE and stained with goat anti-human B7-H3 Ab, after transfer to Immobilon-P. Blots were developed by rabbit anti-goat HRP and ECL. B, RA FLS whole cell lysates were precleared, and then separated on SDS-PAGE, blot transferred, and developed with goat anti-human B7-H3 Ab or goat IgG. Lysates from the same RA FLS line were run in all lanes. These results are representative of two experiments. C, Whole cell lysates from RA FLS, OA FLS, lung fibroblasts, and skin fibroblasts were run on SDS-PAGE without preclearing, blot transferred, and developed with goat anti-human B7-H3 Ab or goat IgG. The film exposure duration was 5 s. Staining is representative of two experiments. Numbers shown to the left of each panel represent molecular mass marker (kDa).
by plates coated with a combination of 10 μg/ml mouse anti-human CD3 and 10 μg/ml mouse anti-human CD28 (TCD3/CD28) for 8 days.

Flow cytometry

Fibroblasts were grown for 48 h in 6- or 12-well plates before stimulation with cytokines: IFN-γ 1000 U/ml, TNF-α 10 ng/ml, IL-1β 1 ng/ml, IL-17 20 ng/ml, IL-10 10 ng/ml, or IL-4 10 ng/ml. Cytokine stimulation proceeded for 72 h. Fibroblasts were removed from plates for staining by incubation with 3 mM EDTA in PBS for 20 min at room temperature. Cells were stained with mouse anti-human Abs specific for CD98, CD54, or B7-H3 (R&D Systems), or with a mouse IgG control. A secondary goat anti-mouse IgG-Alexa Fluor 488 (Molecular Probes) conjugate was used to visualize fluorescence. Cytometry was performed on a Coulter EPICS XL.

Western blot

Western blot analysis used standard protocols. Fibroblasts were grown in 175-cm² flasks to confluence and lysed with a buffer containing 1 mM PMSF, 5 mM iodoacetamide, 8 mM Tris-HCl, 100 mM NaCl, 0.02% NaN₃, and 1% Nonidet P-40 or 1% saponin as the primary detergent. Preclearing was with the following steps: 1) 25 μL of protein G-agarose beads (Invitrogen Life Technologies), 2) 1 μg of mouse IgG (Sigma-Aldrich) followed by 25 μL of protein G-agarose, and 3) 1 μg of goat IgG (Antibodies Inc) followed by two rounds of 25 μL of protein G-agarose. Lysates were run in reducing buffer on Tris-glycine 4–20% acrylamide gradient gels (Novex; Invitrogen Life Technologies) and transferred onto an Immobilon-P membrane (Millipore). Primary Ab, goat anti-human B7-H3 (R&D Systems), was used at a concentration of 1 μg/ml for 1 h at 4°C. Control goat IgG Ab (Sigma-Aldrich) was also used at 1 μg/ml. Membranes were washed three times with wash buffer before development with rabbit anti-goat-HRP conjugate (Molecular Probes) at 0.2 μg/ml for 1 h at 4°C. Membranes were developed by chemiluminescence ECL (Amersham Biosciences).

Immunohistochemistry

RA synovial tissue was fixed in 10% neutral-buffered formalin or frozen in OCT, and sections were mounted onto glass slides. Immunohistochemistry was performed using standard protocols for dichromatic staining of frozen and paraffin-embedded sections (26). Goat anti-human Abs were anti-B7-H3 (R&D Systems), biotinylated anti-B7-H3 (R&D Systems), anti-CD90 (BD Biosciences), and anti-cadherin-11 (R&D Systems). Mouse anti-human Abs were anti-CD3 (R&D Systems), anti-CD68 (DakoCytomation), anti-CD20 (Signet Laboratories), anti-CD45 (Signet Laboratories), and anti-cadherin-11 (Zymed Laboratories). Detection Abs and secondary detection reagents used from Vector Laboratories were biotinylated horse anti-goat IgG, alkaline phosphatase horse anti-mouse IgG, R.T.U. VECTAStain Elite ABC Reagent, Vector Blue Substrate, Vector Red Substrate, and AEC substrate. After staining sections were sealed using Faramount.

Confocal microscopy

FLS were grown on glass coverslips for 48 h. Trest, Tck, or Tsea were cocultured with FLS for various time points. Coverslips were then washed...
with PBS and fixed in 4% paraformaldehyde (Molecular Probes) for 1 h at room temperature or overnight at 4°C. Coverslips were stained using goat anti-human B7-H3 (R&D Systems), mouse anti-human CD11a, and mouse anti-human CD54. Secondary Abs used were goat anti-mouse IgG Alexa Fluor 594, and rabbit anti-goat IgG Alexa Fluor 488. For coculture studies comparing LFA-1 and ICAM-1 localization, FLS were first transfected with an expression vector for a fusion protein of ICAM-1 and enhanced GFP (eGFP), and then plated on glass coverslips. This expression vector was termed CD54-eGFP and was created as follows. Primers were constructed against the sequence described by Accession no. BC015969 to amplify the open reading frame of human ICAM-1 by PCR. A HindIII restriction site added to the beginning of the 5' primer and an AgeI site added to the tail of the 3' primer facilitated ligation of the amplified fragment into the pEGFP-N1 expression vector (Clontech Laboratories). After ligation, the insert sequence was verified to be free of mutation. This resulted in a CD54-eGFP fusion protein expression vector, with ICAM-1 at the N terminus and eGFP at the C terminus (intracellular) and a short peptide linker (GSTPVAT) in between. Transfection was conducted using the Adult Human Dermal Fibroblast kit (Amaxa), and the manufacturer’s protocols were observed. Images were taken using Olympus FluoView 500 Laser Scanning Confocal Microscope.

**B7-H3 knock down and coculture of T cells and FLS**

Three stealth RNA interference (RNAi) and two control RNAi were obtained from Invitrogen Life Technologies algorithms based against the sequence Accession no. AJ583695 from GenBank. Stealth RNAi was pooled in equimolar amounts and used at a working concentration of 10 μM, to

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**FIGURE 4.** (continued)
knock down B7-H3 expression. Control RNAi was also pooled in equimolar amounts and used at a working concentration of 10 μM. FLS were transfected with RNAi using the Adult Human Dermal Fibroblast kit (Amaxa). For transfection, FLS were removed from plates by trypsinization and pelleted. FLS were then resuspended in transfection buffer with 1–5 μM final concentration of RNAi and placed in cuvettes. FLS were electroporated and replated for 48 h before use. As an additional control, FLS were mock transfected without the use of RNAi. Transfected FLS were allowed to rest for 48 h before being seeded onto plates. FLS were left to adhere for an additional 48 h after seeding. To evaluate the effect of FLS on T cell cytokine production, Trest, Tck, or TCD3/CD28 were then added to FLS at a ratio of 1:6.66 FLS to T cells. After 8 days, T cells were collected from cocultures by gentle washing with medium. Harvested T cells were washed twice in medium and then replated at 500,000 cells in 1.5 ml of medium. T cells were then stimulated for 12 h with 10 ng/ml PMA and 0.5 μM ionomycin. Supernatants were harvested, and TNF-α, IFN-γ, and IL-2 levels were measured by ELISA (BD Biosciences).

Results

B7-H3 is expressed by FLS and other fibroblasts

Surface staining of FLS revealed strong expression of B7-H3 by the resting and IFN-γ stimulated cells (Fig. 1A). In contrast, the B7 family member ICOS-L (B7-H2) was not detected (Fig. 1A). Expression of B7-1 or B7-2 (CD80 and CD86, respectively) by FLS was previously reported to be negligible (13, 14). Similar to ICOS-L expression, B7-H1 and PD-L2 expression was negligible on FLS and did not increase with IFN-γ stimulation (Fig. 1B). To assess whether other fibroblasts also selectively expressed B7-H3 and whether immunoregulatory cytokines affected B7-H3 expression, we compared B7-H3 expression on RA FLS, OA FLS, skin fibroblasts, and lung fibroblasts. These various fibroblasts were stimulated, before surface staining, with various immunoregulatory cytokines for 72 h, including IFN-γ, TNF-α, IL-1β, and IL-17. As a control, CD54 (ICAM-1) expression was also measured. All four types of fibroblasts (RA FLS, OA FLS, skin, and lung) expressed high basal levels of B7-H3, which was unaffected by cytokine stimulation (Fig. 1). In contrast, CD54 expression increased or decreased depending on the cell type and cytokine (Fig. 1).

B7-H3 expressed by FLS and other fibroblasts is predominantly four Ig-like domain

Because human B7-H3 can be alternatively spliced to yield 4IgB7-H3 (B7-H3b) or 2IgB7-H3 (B7-H3), Western blot analysis was used to determine the m.w. of B7-H3 expressed by fibroblasts. We first established that the Ab used for Western blot analysis recognized both isoforms of B7-H3, detecting a band at slightly <98 kDa for recombinant 4IgB7-H3 and slightly <50 kDa for 2IgB7-H3 (Fig. 3A).

FIGURE 5. B7-H3 localization at the T cell/FLS synapse. A, FLS were grown on glass coverslips and cocultured with Tseq, Tck, or Trest for 24 h. Coverslips were fixed in 4% paraformaldehyde and stained with goat anti-human B7-H3 (green) and CD3 (red). B. Staining of B7-H3 (green) and CD3 (red) after 2 h of coculture. Images are representative of three experiments.

Whole cell lysate, from RA FLS lysed in Nonidet P-40 was pre-cleared, separated by SDS-PAGE, blot transferred, and probed with B7-H3 Ab. This revealed only one band at ~110 kDa corresponding to glycosylated 4IgB7-H3. Extending the blot development time to 3 h did not reveal the presence of a 2IgB7-H3 band near 50 kDa, but nonspecific background staining was visible (Fig. 3B).

To determine whether OA FLS or fibroblasts from skin and lung also predominantly express 4IgB7-H3, Western blot analysis was again used. Whole cell lysates from RA FLS, OA FLS, skin fibroblasts, or lung fibroblasts were created using saponin as the detergent (which resulted in better recovery of B7-H3 compared...
with Nonidet P-40, data not shown). To minimize any potential loss of 2IgB7-H3 signal, this lysate was not precleared before separation on SDS-PAGE. Blot development yielded a strong band at 110 kDa for all fibroblast types and a much weaker band at ~50 kDa (Fig. 3C). This suggests that the four Ig-like domain isoform is the major and almost exclusive isoform of B7-H3 expressed on fibroblasts.

**B7-H3 is broadly expressed in RA synovium**

To evaluate the expression of B7-H3 in vivo, synovium was obtained from arthroplasty or synovectomy of RA joints, and immediately fixed in formalin for embedding in paraffin, or frozen in OCT. To locate regions rich in FLS, RA synovial sections were stained with the FLS specific anti-cadherin-11, which was compared with B7-H3 staining (Fig. 4A). Cadherin-11 is used by FLS in homotypic adhesive interactions, is expressed by synovial lining FLS, and is important for the overall morphology and development of the synovial lining (27). Monochromatic staining of B7-H3 or cadherin-11 showed almost identical patterns of expression. The strongest localization of cadherin-11/B7-H3 was to the lining layer of RA synovium, however, cadherin-11/B7-H3 was also found diffusely throughout synovial tissue (Fig. 4A). CD90, another fibroblast marker, was used to show the diffuse expansion of FLS in RA synovium, but unlike cadherin-11/B7-H3, CD90 localized most intensely to blood vessel rich regions (Fig. 4A). The immunohistochemical studies indicate that, similar to cultured FLS, the FLS in RA synovium express B7-H3.

Dichromatic immunohistochemical staining was performed, to compare the expression of B7-H3 to immune cell markers. Staining of paraffin sections for B7-H3 and CD20 (B cell), CD45 (hemopoietic), or CD68 (macrophage) surface structures revealed that B7-H3 staining was distinct from CD20 and CD45 (Fig. 4B). There was diffuse B7-H3 expression throughout the pannus with slightly stronger localization toward the lining layer and around blood vessels. Patches of B cells and hemopoietic cells appeared distinct from the B7-H3⁺ cells. The macrophage marker CD68 showed broad staining that overlapped partially with B7-H3 expression. This most likely represents resident macrophage-like synoviocytes and invading macrophages. The control Ab for B7-H3 (goat IgG) displayed only a background staining pattern (Fig. 4B).

Frozen sections were stained to compare B7-H3 staining to CD3 (T cell) or CD80 (APC) staining (Fig. 4C). CD3⁺ T cells were abundant and in close proximity to B7-H3⁺ cells. CD80 staining showed a diffuse pattern, indicating the broad distribution of professional APC within the synovium. However, at the synovial lining, B7-H3 staining was strong and there was little CD80 signal.

By the staining patterns of B7-H3 and CD3, T cells appear to be juxtaposed to FLS throughout RA synovial lesions. However, because B7-H3 is not unique to FLS, CD3 staining was compared with cadherin-11 and CD90 (Fig. 4D). Similar to the pattern seen with B7-H3/CD3 (Fig. 4C), cadherin-11/CD3 and CD90/CD3 staining showed both well-organized and diffuse T cell regions, with many T cells in close proximity to cadherin-11-positive FLS (Fig. 4D).

**B7-H3 localizes to the contact point between FLS and T cells**

T cells representing three different T cell activation states were cocultured with FLS. Tsea were T cells that had been stimulated through their TCR by superantigen presented by FLS. Tck were activated with a cytokine mix (and not by the TCR), consisting of IL-6, TNF-α, and IL-2, and represent a population that share characteristics similar to T cells isolated from RA synovial fluid (25). Trest were T cells that were purified from peripheral blood and not otherwise stimulated. After 2 or 24 h, the cocultures were fixed with paraformaldehyde and stained for B7-H3 and CD3. By 24 h, B7-H3 signal could be seen outlining the contact point of both Tck and Tsea on FLS (Fig. 5). At 24 h, Trest adherence to FLS was marked, but strong B7-H3 signal at the contact site was not clearly visible (Fig. 5A). This localization of B7-H3 to the contact region with Tck and Tsea on FLS is clearly distinct as early as 2 h of coculture (Fig. 5B).

**B7H3 localization at the FLS/Tck point of contact is distinct from the zone of CD54/LFA-1 engagement**

B7-H3 localization was compared with the localization of CD54 (ICAM-1) on FLS and its counter receptor, LFA-1 (CD11a/CD18), on the T cell. FLS were transfected with a CD54-eGFP (green)
expression vector before coculture with Tck. Cocultured cells were fixed and Tck were indirectly stained for CD11a which was visualized by means of the fluorochrome Alexa Fluor 594 (red). At the synapse of Tck and FLS, confocal Alexa Fluor 594 (red). At the synapse of Tck and FLS, confocal imaging revealed merged signals (yellow) between CD54 and LFA-1 (Fig. 6A). In contrast, B7-H3 at the FLS/Tck contact point showed a staining pattern that was distinct from CD54 (Fig. 6B), as seen by the separation of green and red signals. Similarly no merging of B7-H3 and LFA-1 fluorescence was detected (Fig. 6C). The separation of B7-H3 from regions dense in LFA-1/CD54 at the contact point between FLS and Tck could be temporally dependent. It is possible that B7-H3 might migrate to and from LFA-1/CD54 dense areas in a rapid sequence or at an extremely slow rate not detected at the time points selected for confocal imaging. However, we have not observed merged signals of B7-H3 with LFA-1 or CD54 at coculture times up to 24 h (data not shown).

FIGURE 7. B7-H3 specific RNAi knockdown. FLS were transfected with pooled B7-H3 specific RNAi, pooled control RNAi, or mock transfected (with no RNAi) before staining with B7-H3 (black line histogram), CD98 (dashed line histogram), or control mouse IgG (gray-filled histogram) at 12 days post-transfection. Histograms are representative of five experiments.

FIGURE 8. T cell cytokine production after coculture with B7-H3 RNAi transfected FLS. FLS were transfected with pooled B7-H3 RNAi (B7-H3), pooled control RNAi (Control), or mock transfected (No RNA) and cocultured with Trest, Tck, or TCD3/CD28. T cells were harvested after 8 days of coculture and restimulated with PMA and ionomycin. Supernatants were measured for cytokines by ELISA. Error bars represent 95% confidence intervals. Data are representative of three experiments. A, TNF-α production. Using two-tailed t test analysis comparing cytokine production from Trest cultured with B7-H3− FLS (B7-H3) to B7-H3+ FLS (Control) and with B7-H3− FLS (B7-H3) to mock transfected (No RNA), yielded p values <0.0021 and <2.2 × 10⁻³, respectively. Similarly, t test analysis for Tck yielded p values <9.1 × 10⁻⁶ and <5.6 × 10⁻⁷, respectively. B, IFN-γ production. Trest cytokine production yielded p values <1.8 × 10⁻⁵ and <0.0013, respectively. Tck cytokine production yielded p values of <2.8 × 10⁻⁹ and <1.2 × 10⁻⁷, respectively. C, IL-2 production. Trest cytokine production yielded p values <2.5 × 10⁻³ and <0.0005, respectively. Tck cytokine production yielded p values of <2.4 × 10⁻⁵ and <2.7 × 10⁻⁶, respectively.
Distinct functions of B7-H3 in interactions with Trest vs Tck

To assess the role of B7-H3 in cocultures of T cells and FLS, RNAi technology was used to knock down B7-H3 expression in FLS before coculture with T cells. Pools of specific or control RNAi were transfected into FLS and screened for effective inhibition of B7-H3. Effective and specific knock down of B7-H3 persisted for at least 12 days post-transfection (Fig. 7). RNAi transfection did not adversely affect CD98 expression in mock transfected or RNAi transfected FLS (Fig. 7).

The following T cells were representative of three activation states cocultured with FLS: Trest were freshly isolated from peripheral blood; Tck were similar to RA synovial T cells (25); TCD3/CD28 were T cells that had been previously activated through plate-bound anti-CD3/anti-CD28. These were used in place of Tsea to represent TCR-stimulated T cells in these experiments, to avoid the need for exposure of T cells to APC before incubation with B7-H3 knockdown FLS, which could potentially confound interpretation of the results. These various types of T cells were cocultured with FLS that had been transfected with pooled B7-H3 RNAi, pooled control RNAi, or mock transfected FLS (no RNAi). After 8 days of coculture with B7-H3 knockdown FLS, T cells were harvested and restimulated with PMA and ionomycin for 12 h. Production of TNF-α, IFN-γ, and IL-2 by the T cells was measured by ELISA. Although cytokine production by TCD3/CD28 following coculture with FLS was not substantially affected by B7-H3 knockdown, striking and opposite effects on cytokine production of Tck and Trest were observed. Trest cocultured with B7-H3− FLS showed increased levels of cytokine production compared with FLS transfected with control RNAi (Fig. 8). In contrast, Tck cocultured with B7-H3− FLS showed reduced cytokine production compared with coculture with control RNAi FLS (Fig. 8). This suggests that B7-H3 has an inhibitory effect on Trest and a stimulatory effect on Tck.

Discussion

B7-H3 is inhibitory or costimulatory

To achieve full activation during the response to peptide Ags, T cells require two stimulatory signals. TCR engagement of MHC/Ags triggers complex signaling cascades in T cells, but alone this is insufficient for activation (28–31). A costimulatory signal is required in conjunction with TCR signaling to induce clonal proliferation and prevent anergy. This additional signal to the TCR is typically provided by a surface structure present on accessory cells, such as B7-1 (CD80), which can engage the CD82 molecule on the T cell surface (32–36). The discovery of a second receptor for B7, CTLA-4, which is important in limiting T cell responses, established a molecular basis for the bifunctional role of B7 as both an activating and inhibitor ligand (37).

Starting from the initial B7-1 costimulatory ligand, a family of B7 molecules has been discovered. B7-2 (B70, CD86) is a second ligand for both CD28 and CTLA-4. New ligand/receptor pairs include PD-L1 (B7-H1)/PD-1, PD-L2 (B7-DC)/PD-1, ICOS-L (B7-H2, B7h, B7RP-1)/ICOS, and B7-H4/BTLA (38–40). These novel B7 family molecules have documented effects in enhancing T cell functions, inhibiting T cell functions, or both.

This duality is further reinforced by the more recent characterization of the B7-related protein VSIG4. Initially, VSIG4 (CR1g) was described as a complement receptor necessary for binding enzymatic products of C3 and for effective phagocytosis by Kupffer cells (41). In addition, VSIG4 has been also been found to reduce T cell proliferation by CD3/CD28 stimulation in vitro, and to blunt CD8 T cell responses and IFN-γ production in vivo (42). This complex pattern of inhibition and/or activation is an important characteristic of the B7 family. Thus beyond the their role in immune activation, the B7 family functions in the complex realm of immune modulation (38–40). This is exemplified by the discoveries that CD28 and CTLA-4 not only transduce signals into the T cell by ligation of B7 family members, but that the signaling is bidirectional with functional signals transmitted into the APC as well (43, 44). Further complicating immune modulation by B7 family members is the more recent discovery that PD-L1 and B7-1 can indeed bind to each other, and that this interaction is inhibitory and signals bidirectionally (45).

The current study has documented expression of a B7 family costimulatory molecule, B7-H3, on FLS and other fibroblasts, which are not typically viewed as professional APC. Unlike professional APC, which only express B7-H3 after activation (15), FLS expression is constitutive and uninfluenced by immunoregulatory cytokines. This constitutive B7-H3 expression is overwhelmingly of the Ig-like domain isoform and is robust both in vivo and in vitro.

The literature on the effects of B7-H3 on T cells has not hitherto provided consensus as to its function. B7-H3 was originally described in human systems as a two Ig-like domain costimulatory molecule that increased T cell proliferation, enhanced cytotoxicity, and increased IFN-γ production (15). However, a subsequent study indicated that the predominant isoform of B7-H3 is the four Ig-like domain form (17). This report did not recapitulate the costimulatory effects originally documented. In yet another study, NK lysis of neuroblastoma lines was enhanced by blocking Abs against 4lg B7-H3 (46).

Studies of B7-H3 in mice, which only contain one isoform, the two Ig-like domain isoform (16, 18), do not resolve this issue. B7-H3 knockout mice showed increased airway hypersensitivity and earlier onset of experimental autoimmune encephalomyelitis (20). Similar results were obtained using B7-H3 blocking Abs, notably that T cells increased cytokine production and experimental autoimmune encephalomyelitis symptoms were exacerbated by B7-H3 blockade (19). In contrast to reports in humans, tumor expression of B7-H3 enhances cytotoxicity (21, 22). And when B7-H3 knockout mice are used in allograft survival studies, B7-H3-deficient transplants fare better when combined with immunosuppression (23). Thus far, there appears to be no specific and consistent association of the two Ig-like or four Ig-like B7-H3 domain with activating or inhibitory effects.

We found that B7-H3 expression was associated with FLS rich areas and was in close proximity to T cells in the RA pannus. When the T cell-FLS interaction was explored by confocal microscopy, B7-H3 was found at the contact point, but was differentially localized from CD54 and LFA-1. This suggested that B7-H3 could be a significant signaling molecule between FLS and T cells. However, knock down of B7-H3 in FLS did not affect the ability of FLS to present superantigen to T cells or to respond to contact with T cells by the secretion of IL-6 and IL-8 (data not shown). Nevertheless, interesting B7-H3 dependent effects on cytokine secretion by T cells following coculture with FLS were documented, including the differential effects of B7-H3 on cytokine production by resting or Tck cells. This suggests that the activation state of the T cell has significant influence over the outcome of T cell ligation of B7-H3, and also suggests that there may be two receptors for B7-H3 (which is a pattern that would be similar to other B7 family ligands). Furthermore, cytokine production by TCD3/CD28 did not greatly differ after coculture with B7-H3 expressing or knockdown FLS.

A simplistic model to explain our observations would be that Trest predominately express an inhibitory receptor for B7-H3,
whereas Tck predominantly express an activating receptor for B7-H3. T cells activated through stimulation by CD3 and CD28 did not have significant differences in cytokine production following B7-H3 knockdown, suggesting that this type of activated T cells does not express a functionally significant level of B7-H3 receptor, or that, perhaps, opposing effects of engagement of both positive and negative receptors are offsetting. So far we have not observed an effect of engagement or interference with B7-H3 signaling on FLS activation, implying that perhaps B7-H3 signaling is not bi-directional in FLS-T cell interactions as compared with the roles of various B7 family molecules in interactions of T cells with professional APC. However, an alternative possibility is that other assays need to be used to measure the functional consequences of B7-H3 engagement, or inhibition, on the B7-H3 expressing cells.

**Costimulation in RA**

The strong expression of B7-H3 by FLS of the RA pannus raises interesting issues regarding costimulation of T cells in RA. It has been suggested that an unusual subset of CD4+ CD28null T cells is expanded in RA (47, 48). These T cells are likely to require co-stimulatory signals distinct from CD80/CD86. There is evidence that constant exposure to TNF-α (which is abundant in RA) blocks the transcription of CD28 (49). These CD4+ CD28null T cells have been shown to be potent producers of cytokines (including TNF-α and IFN-γ) (50), and to also express costimulatory molecules normally associated with NK cells (51). It will be interesting to determine whether this CD4+ CD28null T cell subset interacts with FLS through the B7-H3 molecule.

CD4+ CD28null T cells are, of course, not the only inflammatory T cell subset in RA. Recent trials indicate that the drug abatacept (CTLA-4-IgG fusion protein) has clinical benefit in RA (52). Un-


