Identification of an Interleukin 17F/17A Heterodimer in Activated Human CD4+ T Cells**

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IL-17F and IL-17A are members of the IL-17 pro-inflammatory cytokine family. IL-17A has been implicated in the pathogenesis of autoimmune diseases. IL-17F is a disulfide-linked dimer that contains a cysteine-knot motif. We hypothesized that IL-17F and IL-17A could form a heterodimer due to their sequence homology and overlapping pattern of expression. We evaluated the structure of recombinant IL-17F and IL-17A proteins, as well as that of natural IL-17F and IL-17A derived from activated human CD4+ T cells, by enzyme-linked immunosorbent assay, immunoprecipitation followed by Western blotting, and mass spectrometry. We find that both IL-17F and IL-17A can form both homodimeric and heterodimeric proteins when expressed in a recombinant system, and that all forms of the recombinant proteins have in vitro functional activity. Furthermore, we find that in addition to the homodimers of IL-17F and IL-17A, activated human CD4+ T cells also produce the IL-17F/IL-17A heterodimer. These data suggest that the IL-17F/IL-17A heterodimer may contribute to the T cell-mediated immune responses.

Interleukins 17F (IL-17F) and 17A (IL-17A) are closely related members of the IL-17 cytokine family, and share 50% amino acid identity. Studies in the mouse have identified Th17 cells as a distinct CD4+ T cell lineage that is defined by the production of IL-17F and IL-17A (1–7). IL-6 and transforming growth factor-β (TGF-β) are required for the differentiation of naïve CD4+ T cells to Th17 cells (1, 8), which are maintained in the presence of IL-23 and IL-1β. Conversely, IL-4 and interferon-γ can inhibit the development of Th17 cells (9, 10). Th17 cells have been implicated in the pathology of mouse autoimmune disease models (2).

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3 The abbreviations used are: IL, interleukin; TGF-β, transforming growth factor-β; ELISA, enzyme-linked immunosorbent assay; nano-LC-MS/MS, nano-liquid chromatography-tandem mass spectrometry; PBS, phosphate-buffered saline; ACN, acetonitrile; FA, formic acid; CM, conditioned medium; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Expression of IL-17F and IL-17A has been detected in activated human peripheral blood lymphocytes. It has been shown by reverse transcriptase-PCR experiments that the cytokines are expressed in activated human CD4+ T cells (11, 12). Expression of IL-17F and IL-17A has also been observed in tissue samples from various autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, psoriasis, inflammatory bowel disease, and asthma (2, 3, 13–22).

The crystal structure of IL-17F has been solved and shows that the protein forms a disulfide-linked dimeric glycoprotein (23). IL-17A is also a disulfide-linked homodimeric glycoprotein (24), although crystal structure or data defining the precise subunit interactions are lacking. The IL-17F homodimer includes a classical cysteine knot motif, which is found in the TGF-β, bone morphogenetic protein, and nerve growth factor superfamilies (25, 26). One difference in the cysteine knot motif of IL-17F compared with the other known cysteine knot protein families is that it only utilizes four cysteines instead of the classical six cysteines to form the knot.

There have been reports that some of the cysteine knot family members can exist as heterodimers in vivo. TGF-β1 and -β2 were identified in bovine bone extracts, whereas inhibin and activin AB have been found in gonadal fluids (27–29). In addition, there are other cytokines that are expressed as heterodimers, such as IL-12, which is composed of two protein subunits p35:p40 (30), and the related cytokine, IL-23, which is composed of p19:p40 protein subunits (31).

Here we find that co-expression of IL-17F and IL-17A in HEK293 cells results in the production of the biologically active IL-17F/IL-17A heterodimer, in addition to the IL-17F and IL-17A homodimers. More importantly, we show for the first time that activated human CD4+ T cells produce the IL-17F/IL-17A heterodimer along with the corresponding homodimers.

EXPERIMENTAL PROCEDURES

Cloning of IL-17 Proteins—Full-length IL-17F and IL-17A genes were cloned from phorbol 12-myristate 13-acetate and the ionomycin-activated human peripheral blood mononuclear cell library and used as templates for the following constructs. The amino-terminal FLAG™-tagged IL-17A construct was made by sequential overlapping PCR amplifications using a honeybee signal sequence, followed by a GSP spacer, and a FLAG tag (DYKDDDDK) joined to the mature region of IL-17A (residues 20–156). The amino-terminal streptavidin- and FLAG-tagged IL-17A construct consists of a honeybee signal...
sequence, a GSP spacer, the streptavidin tag (WSHPQFEK), a GSG spacer, and the FLAG tag (DYKDDDDK) joined to the mature region of IL-17A (residues 20–156). The amino-terminal His-tagged IL-17F construct consists of a honeybee leader, a GSG spacer, a His tag, and an EK cleavage site (GDDDDK) fused to mature IL-17F (residues 31–164). The amino-terminal protein C (HPC)-tagged IL-17F constructs consist of a honeybee leader, a GSG spacer, a HPC (EDQVDPRLIDGK) tag, an GSG spacer, a His tag followed by an EK cleavage site and fused to mature IL-17F (residues 31–164). All constructs utilized a cytomegalovirus promoter driven expression vector and were verified by DNA sequencing.

Expression of IL-17 Proteins—Proteins were expressed by transient transfection (TransIT-LT1, Mirus) of HEK293 cells (for purification) or COS-1 cells (for immunoprecipitation). Heterodimers were produced by co-transfection of IL-17F and IL-17A on separate plasmids using equal amounts of DNA. Twenty-four hours post-transfection, medium containing the DNA/liposome mixture was removed and replaced with serum-free medium. The conditioned medium was harvested 48 h later and protein production was evaluated by Western analysis.

Purification of IL-17 Proteins—Chinese hamster ovary conditioned medium containing the His-tagged IL-17F homodimer was passed over a nickel-NTA Fast Flow column (Qiagen). The column was washed with 17.5 mM imidazole and eluted with 250 mM imidazole. The eluted protein was dialyzed against PBS (pH 7.2), and then digested with enterokinase at a 1500:1 ratio of protein to enterokinase for 4 h at room temperature. The digested protein was re-applied to nickel-NTA and washed with 10 mM imidazole. The cleaved IL-17F was eluted with 40 mM imidazole. HEK293 conditioned medium containing the streptavidin-FLAG-tagged IL-17A homodimer was flowed over a Strept-Tactin column (IBA), then washed with Tris-buffered saline and eluted with 3 mM desthiobiotin. HPC His-tagged IL-17F/FLAG-tagged IL-17A heterodimer was purified by batch binding the co-transfected HEK293-conditioned medium to an anti-FLAG M2 affinity resin (Sigma). The IL-17A homodimer and IL-17F/IL-17A heterodimer were eluted with 200 μg/ml of FLAG peptide (Sigma). The protein was then batch bound to an anti-protein C affinity matrix (Roche) and the heterodimer was eluted with 5 mM EDTA. All of the purified proteins were dialyzed against PBS (pH 7.2), and characterized by SDS-PAGE gel, Western analysis, mass spectrometry, and analytical SEC.

ELISA Quantitation of IL-17 in Conditioned Medium—The IL-17A homodimer concentration was determined by capturing with plate-bound anti-huIL-17A antibody (4 μg/ml of clone 41809; R&D Systems) followed by detection with the same antibody (0.5 μg/ml) and poly-streptavidin horseradish peroxidase (1/10,000; Pierce). Recombinant human IL-17A homodimer (Wyeth Research) was used as a standard. Due to the antibody selection based on specificity, the IL-17A ELISA sensitivity is 2.5 ng/ml. The concentration of IL-17F homodimer was determined by capturing with plate-bound anti-huIL-17F-07 (2 μg/ml; Wyeth Research) followed by detection with biotinylated anti-huIL-17F-07 (0.5 μg/ml) and streptavidin horseradish peroxidase (1/10,000; Zymed Laboratories Inc.), using recombinant human IL-17F as a standard (Wyeth Research). IL-17F/A heterodimer concentration was determined by capturing with plate-bound anti-huIL-17F-01 (2 μg/ml; Wyeth Research) followed by goat anti-huIL-17A antibody (75 ng/ml; R&D Systems) and streptavidin horseradish peroxidase (1/10,000; Zymed Laboratories Inc.), using recombinant human IL-17 F/A heterodimer (Wyeth Research) as a standard.

Immunoprecipitation of IL-17 for Western Blot—COS conditioned medium (50 μl) was mixed with 20 μg/ml murine anti-huIL-17A-02 (Wyeth Research) or murine anti-huIL-17F-01 (Wyeth Research) monoclonal antibodies for 1 h at 4 °C under gentle rotation. Antibody complexes were captured with 50 μl of hydrated Protein A-Sepharose (Amersham Biosciences) overnight at 4 °C under gentle rotation. The beads were then sequentially washed with PBS + 1% Tween 20, PBS + 0.1% Tween 20, and PBS + 0.05% Tween 20. The immunoprecipitated pellets were resuspended in non-reducing sample buffer and loaded onto a 10% Tricine gel for Western blot analysis using either goat anti-huIL-17A (R&D Systems) or rabbit anti-huIL-17F antibodies (Wyeth Research) for detection.

In Vitro T Cell Activation for Immunoprecipitation followed by Western Blot—Human CD4+ T cells from peripheral blood lymphocytes were purified using RosetteSep® ( StemCell Technologies Inc). The cells were stimulated for 48 h with anti-CD3 microspheres and soluble anti-CD28 at 5 μg/ml. After this primary activation, the microspheres were removed and the cells were washed, resuspended in fresh medium, and rested overnight. The rested cells underwent a secondary stimulation with the same amounts of anti-CD3 microspheres and soluble anti-CD28 in addition to 60 ng/ml huIL-21 (R&D Systems). The supernatant was harvested at 72 h.

Immunoprecipitation of IL-17 from Activated CD4+ T Cells—Activated T cell medium (500 μl) was mixed with 15 μg/ml murine anti-huIL-17A-02 (Wyeth Research) or murine anti-huIL-17F-01 (Wyeth Research) monoclonal antibodies and treated as described above for the immunoprecipitation of COS conditioned medium. The immunoprecipitated pellets were resuspended in non-reducing sample buffer and loaded onto a 10% Tricine gel for Western blot analysis using a rabbit anti-huIL-17F antibody for detection (Wyeth Research).

In Vitro T Cell Activation for ELISA and Mass Spectrometry—Human CD4+ T cells were purified as described above. Tosyl-activated magnetic microspheres (Dynal Biotech) were coated with anti-CD3 antibody (clone UCHT-1, 1 μg/10⁷ microspheres) and anti-CD28 (clone CD28.2, 0.5 μg/10⁷ microspheres). Human IgG was used to saturate the binding capac-
ity of the microspheres (total protein = 5 μg/10^7 microspheres). Protein-coated microspheres were added to purified CD4+ T cells (1 x 10^6 cells/ml) at a ratio of 1:1. Additionally, 10 ng/ml IL-23, 1 ng/ml TGF-β, 20 ng/ml IL-6, 10 ng/ml TNF-α, 10 ng/ml IL-1β, 10 μg/ml anti-IL-4, and 1 μg/ml anti-interferon γ were added to the culture. Supernatants (90 ml at 2 x 10^6 cells/ml) were harvested at 72 h and the concentrations of homodimeric and heterodimeric IL-17F and IL-17A were measured by ELISA.

**Mass Spectrometry of IL-17 from Activated CD4+ T Cells and COS CM—**70 ml of activated human T cell medium was mixed with 15 μg/ml murine anti-IL-17A-02 (Wyeth Research) and 20 μg/ml murine anti-huIL-17F-01 (Wyeth Research) antibodies for 1 h at 4°C under gentle rotation. Antibody complexes were captured with 175 μl of hydrated Protein A-Sepharose (Amersham Biosciences) overnight at 4°C under gentle rotation. The immunoprecipitated pellets were then sequentially washed with PBS + 1% Tween 20, PBS + 0.1% Tween 20, and PBS + 0.05% Tween 20. The immunoprecipitated proteins from activated T cells or purified recombinant proteins (1–2 μg) were eluted from the pellets using SDS sample buffer and then loaded onto a 10–20% Tricine gel (Invitrogen). The proteins were stained with Imperial™ Protein Stain Solution (Pierce). The positively stained gel bands were excised and further cut into about 1 x 1-mm^2^ pieces. To remove the stain, the gel pieces were dehydrated in acetonitrile (ACN), rehydrated, and washed in 25 mM sodium phosphate (pH 6.0), and dehydrated again in ACN. Proteins were digested by immersing the gel pieces in a solution containing 0.5 μg of trypsin in 25 mM sodium phosphate (pH 6.0) at 37°C for 4 h. The resultant peptides were extracted from the gel using 60% ACN + 1% formic acid (FA) and 90% ACN + 5% FA, successively. All eluted fractions were then combined for each sample, concentrated to near dryness by vacuum and reconstituted again in ~20 μl of 2% ACN and 0.1% FA prior to mass spectrometric analysis.

Mass spectrometric analysis was performed on an Agilent 1100 nanoflow system connected to a linear ion trap mass spectrometer (LTQ, ThermoFinnigan). The digested samples were pressure-loaded onto a C18 PicoFrit microcapillary column (New Objective) packed with Magic C18 beads (5 μm, 75 μm × 11 cm, Michrom BioResources) and desalted on-line with solvent A (2% ACN and 0.1% FA) prior to mass spectrometric analyses. The peptides were eluted with a gradient from 4 to 60% solvent B (90% ACN and 0.1% FA) over 70 min with a flow rate of 250 nl/min. The fragment ion spectra (MS/MS spectra) were acquired in a data-dependent manner in which each MS scan was followed by consecutive MS/MS scans on the first three most intense ions from the MS scan. To gain additional sensitivity, the ions with m/z values corresponding to the hypothesized disulfide-bonded peptides were specifically targeted using selected ion monitoring (m/z window 10 Da). The fragment ion spectra of all disulfide-linked peptides were man-
IL-17F/A Heterodimer

TABLE 1

Mass spectrometric analysis of disulfide-linked peptides from the recombinant cytokines

List of inter-protein disulfide-linked peptides identified from the purified recombinant IL-17F and IL-17A homodimers as well as the IL-17F/IL-17A heterodimer. The peptide sequences have been confirmed by their corresponding parent ion masses and fragment ion spectra acquired by nano-LC-MS/MS. Peptide portions from IL-17F and IL-17A are highlighted in bold and italics, respectively.

| Structure          | Theoretical molecular weight | m/z calculated [M + 3H]^3+ recombinant | m/z observed [M + 3H]^3+ |
|--------------------|------------------------------|----------------------------------------|--------------------------|
| IL-17A             | 2754.2                       | 919.1                                  | 919.6                    |
| IL-17FA-1          | 3409.6                       | 1137.5                                 | 1138.1                   |
| IL-17FA-2          | 2419.1                       | 807.4                                  | 807.9                    |
| IL-17F             | 3588.7                       | 1197.2                                 | 1196.9                   |

FIGURE 3. Fragment ion spectrum of an interdimer disulfide-linked peptide resulting from tryptic digestion of non-reduced recombinant IL-17A homodimer. The parent peptide ion is charged [M + 3H]^3+ and has an m/z at 919.64. Experimentally detected sequence ions (b or y ions) are underlined. The ions marked with the asterisk are water-loss fragments.

uually interpreted based on the IL-17F and IL-17A protein sequences. To further confirm the presence of the disulfide linkages, part of the immunopurified IL-17 proteins were also reduced with 100 mM dithiothreitol for 1 h at 37 °C and then alkylated using 113 mM iodoacetamide in 100 mM Tris-HCl buffer (pH 8.5) for 30 min at room temperature in the dark, prior to in-gel tryptic digestion and subsequent mass spectrometric analysis.

RESULTS

Plasmids containing either IL-17F or IL-17A cDNA or both were transfected into COS cells. Cytokine expression in the conditioned medium was determined by ELISA analysis, using capture and detection antibodies that were specific for either IL-17F or IL-17A or IL-17F/IL-17A heterodimer (see supplementary data). As shown in Fig. 1, the IL-17F or IL-17A homodimer could be detected in the conditioned medium following transfection with either cDNA using antibodies specific for either cytokine (A/A; F/F). Co-transfection of both plasmids resulted in the secretion of both IL-17F and IL-17A homodimers (A/A; F/F), as well as a protein that could be captured using an anti-huIL-17F antibody and detected with the anti-huIL-17A antibody (F/A) or the reciprocal capture and detection pair (A/F), consistent with an IL-17F/IL-17A heterodimer.

The transfected COS cell-conditioned medium was further characterized by immunoprecipitation followed by Western blot analysis. When an anti-huIL-17A-specific antibody was used for both immunoprecipitation and Western detection on medium from COS cells transfected with only IL-17A, two
bands are detected that migrate between 25 and 37 kDa that are consistent with the glycosylated forms of the IL-17A homodimer (Fig. 2A, lane 3) (32). These bands are not seen using this antibody on conditioned medium from COS cells expressing only IL-17F (Fig. 2A, lane 2). The bands detected above the 50-kDa molecular mass marker are the heavy and light chains of the antibodies used in the immunoprecipitation. When an anti-huIL-17F-specific antibody was used for both the immunoprecipitation step and Western detection on medium from COS cells transfected with only IL-17F, two bands are detected that migrate at or just above 37 kDa that are consistent with the glycosylated forms of the IL-17F homodimer (Fig. 2B, lane 3). These bands are not seen using this antibody on conditioned medium from COS cells expressing only IL-17A (Fig. 2B, lane 2).

When an anti-huIL-17F-specific antibody was used for the immunoprecipitation and an anti-huIL-17A antibody was used for Western detection, two bands were detected in the co-transfected conditioned medium, which were not seen in the singly transfected medium (Fig. 2A, lanes 5–7). The upper band is the IL-17F homodimer (due to the cross-reactivity of the detection antibody with IL-17F at high protein concentrations in Western blots, data not shown) and the lower band is the IL-17F/IL-17A heterodimer. When immunoprecipitation was carried out using an anti-huIL-17A specific antibody and an anti-huIL-17F antibody was used for Western detection, a band corresponding to the IL-17F/IL-17A heterodimer was confirmed by mass spectrometric analyses of the purified recombinant proteins. The purified IL-17F or IL-17A homodimers and heterodimer were first analyzed by nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) to determine the exact inter-protein disulfide-linked peptides within IL-17A. A number of the disulfide-linked peptides were identified based on their corresponding fragment ion spectra. For example, a peptide with m/z at 1196.9 (3+) corresponds to an inter-protein disulfide-linked peptide from the IL-17F homodimer, with Cys\(^{\text{17}}\) from one of the IL-17F molecules covalently linked to Cys\(^{\text{107}}\) from the other IL-17F molecule (Table 1). This linkage is consistent with the findings from the crystal structure of the IL-17F homodimer (23). A similar disulfide-linked peptide for the IL-17A homodimer was also positively identified. The fragment ion spectrum of this peptide is shown in Fig. 3, in which the sequence ions (b and y ions) clearly indicate an inter-protein disulfide bond between residues Cys\(^{\text{10}}\) and Cys\(^{\text{108}}\). To our knowledge, this is the first report of the inter-protein disulfide-linked peptides within IL-17A.

NH\(_2\)-terminal sequencing and Western blot analysis indicated that the purified IL-17F/IL-17A heterodimer contained both IL-17F and IL-17A sequences. Mass spectrometric analysis identified that the IL-17F/IL-17A heterodimer contains two inter-protein disulfide-linked peptides, each with one peptide from IL-17F and the other from IL-17A. These peptides were identified based on their molecular masses (Table 1) and fragment ion spectra (data not shown). This clearly confirms that COS cells are capable of expressing an IL-17F/IL-17A heterodimer. In addition, the positions of the disulfide linkages (Cys\(^{\text{17}}\)(F)-Cys\(^{\text{106}}\)(A) and Cys\(^{\text{107}}\)(F)-Cys\(^{\text{10}}\)(A)) from the het-

![Image](http://example.com/image.png)

**FIGURE 4. Activity of the cytokines on BEAS-2B cells.** BEAS-2B cells were seeded at 2 × 10⁴ cells/well into 96-well microtiter plates containing serial dilutions of the purified cytokines: IL-17A, IL-17F, and IL-17F/IL-17A. The supernatants were analyzed for GRO-α by ELISA.

| Donor | IL-17A/A | IL-17F/A | IL-17F/F |
|-------|----------|----------|----------|
|       | ng/ml    |          |          |
| 147   | <2.5     | 30       | 39       |
| 215   | <2.5     | 3.5      | 7        |
| 281   | <2.5     | 18.4     | 22       |
| 339   | <2.5     | 8.4      | 14       |
| 353   | <2.5     | 6.4      | 12       |
| 366   | <2.5     | 11.1     | 27       |

**TABLE 2** IL-17 ELISAs on activated human CD4+ T cell CM

Human CD4+ T cells were purified from blood lymphocytes by RosetteSep®. The CD4+ T cells were stimulated by adding beads coated with anti-CD3 (1 μg/10⁷ microspheres) and anti-CD28 (0.5 μg/10⁷ microspheres), 10 ng/mL IL-23, 1 ng/mL TGF-β, 20 ng/mL IL-6, 10 ng/mL TNF-α, 10 ng/mL IL-1β, 10 μg/mL anti-IL-4, and 1 μg/mL anti-IFNγ. The supernatants from six donors were evaluated for the presence of IL-17F and IL-17A homodimers and heterodimer by ELISA.

**FIGURE 5. Activated human CD4+ T cells produce IL-17F/A heterodimers.** Immunoprecipitation followed by non-reducing SDS-PAGE and Western blot on 500 μl of activated human CD4+ T cell CM (lanes 3 and 4), or 50 ng of purified IL-17F spiked into CM (lane 2) or 100 ng of purified IL-17A homodimer spiked into CM (lane 5). Lane 1, molecular weight markers; lanes 2, 3, and 5, immunoprecipitate (IP) with anti-huIL-17F; or lane 4, anti-huIL-17A and detection with anti-huIL-17F. The arrows represent the area in which the three cytokines migrate on an SDS-PAGE gel.
IL-17F/A Heterodimer

The activity of purified IL-17F, IL-17A, and the IL-17F/IL-17A heterodimer was evaluated using a cell-based assay. It has been shown that IL-17F induces the secretion of IL-8 and the granulocyte/macrophage colony-stimulating factor in the BEAS-2B human bronchial epithelial cell line (17, 33). ELISA analysis of conditioned medium from BEAS-2B cells cultured with IL-17F, IL-17A, or the IL-17F/IL-17A heterodimer showed that all three cytokines can also induce GRO-α secretion, and that IL-17F was less potent than IL-17A in the induction of GRO-α secretion. The IL-17F/IL-17A heterodimer was found to be less potent than IL-17A but more potent than IL-17F (Fig. 4). These data indicate that the purified recombinant IL-17F and IL-17A homodimers are biologically active, as is the IL-17F/IL-17A heterodimer.

To determine which forms of IL-17F and IL-17A human T cells produce, CD4+ T cells from six donors were activated with anti-CD3 plus anti-CD28 under conditions reported to induce the production of IL-17A (as described under “Experimental Procedures”). ELISA analysis of the conditioned medium from six donors indicated that secretion of the IL-17F homodimer is greater than that of the IL-17F/IL-17A heterodimer, whereas the IL-17A homodimer is the least abundant with most of the donors’ expression levels being below the detection of the assay, 2.5 ng/ml (Table 2).

Activated human CD4+ T cell conditioned medium was generated and characterized by immunoprecipitation and Western blot analysis. When an anti-huIL-17F antibody was

FIGURE 6. A, Coomassie Blue-stained gel image of the partially purified IL-17F/A heterodimer from conditioned medium. Major bands A, B, and C correspond to IL-17F homodimer, IL-17F/A heterodimer, and IL-17A homodimer, respectively. B and C, fragment ion spectra of two interdimer disulfide-linked peptides resulting from trypsin digestion of the IL-17F/A heterodimer purified from human CD4+ T cell conditioned media. B, tryptic peptide that contains Cys17(IL-17F)-Cys106(IL-17A) linkage with parent ion mass at m/z 1138.32 ([M+3H]+, triply charged). C, tryptic peptide that contains the Cys107(IL-17F)-Cys10(IL-17A) linkage with parent ion mass at m/z 807.81 ([M+3H]+, triply charged). Experimentally detected sequence ions (b or y ions) are underlined. The ions marked with an asterisk and number sign are water-loss and ammonium-loss fragments, respectively.
used for both immunoprecipitation and the Western blot, a band was detected that co-migrated with recombinant IL-17F homodimer that was immunoprecipitated from COS-1 conditioned medium using the same method (Fig. 5, lane 2), suggesting that the band from the activated CD4+ T cell conditioned medium corresponds to the IL-17F homodimer (Fig. 5, lane 3). However, the IL-17F/IL-17A heterodimer was not detected in this format, presumably because of insufficient sensitivity of the antibody used for immunoprecipitation. When an anti-huIL-17A antibody was used for immunoprecipitation and an anti-huIL-17F antibody was used for detection, a band migrating lower than the IL-17F homodimer on the Western blot was observed that is consistent with the molecular weight of the IL-17F/IL-17A heterodimer (Fig. 5, lane 4). The bands detected running just above 50 kDa are the heavy and light chains of the antibodies used in the immunoprecipitation.

To obtain more direct physicochemical evidence for the presence of the IL-17F/IL-17A heterodimer in activated human CD4+ T cell medium, the conditioned medium (70 ml) was immunoprecipitated with a combination of anti-huIL-17F and anti-huIL-17A antibodies. The resulting complexes were captured by protein A-Sepharose beads and separated by SDS-PAGE (Fig. 6A). Bands migrating between 25 and 50 kDa were cut out of the gel and subjected to in-gel trypsin digestion and subsequent nano-LC-MS/MS analysis. Peptides from only IL-17F or IL-17A were identified in bands A and C, respectively. These peptides include the previously identified interdimer disulfide-linked peptides from the recombinant IL-17F or IL-17A homodimers. However, a number of peptides from both IL-17F and IL-17A were identified from band B (data not shown). More importantly, using the selected ion monitoring technique, we were able to obtain two robust fragment ion spectra that correspond to two interdimer disulfide-linked peptides from the IL-17F/IL-17A heterodimer (Fig. 6, B and C). In Fig. 6B, all the detected sequence ions before Cys17 from the IL-17F unit (b4–11, y3–7, and y9) remain unchanged (consistent with the sequence ions from the linear peptide sequence VGHTFFQPSCPVPVGSMK, 6–26), whereas all the sequence ions on and after Cys17 (b15, b19–20, y11, y13–15, y17–18, and y20) have a mass shift of 1180.53 Da, which is equal to the mass of a peptide (EPPHCPSFR, 102–111) from the IL-17A subunit. On the other hand, the sequence ions for peptide EPPHCPNSFR also indicate a mass addition of 2227.08 at Cys106 within the IL-17A subunit and this mass corresponds to a disulfide-linked IL-17F peptide (VGHTFFQPSCPVPVGSMK, 6–26). These fragment ion patterns clearly demonstrate that there is an intersubunit disulfide linkage between Cys17 from IL-17F and Cys106 from IL-17A. The presence of another intersubunit disulfide bond between Cys107 (IL-17F) and Cys10 (IL-17A) can also be clearly confirmed in a very similar manner based on its corresponding fragment ion spectra (Fig. 6C). In addition, these two fragment ion spectra were identical to those observed for the purified recombinant IL-17F/A heterodimer (data not shown). Thus we have confirmed that not only are the IL-17F and IL-17A homodimers expressed by activated human primary CD4+ T cells, so is the IL-17F/IL-17A heterodimer.

**DISCUSSION**

In this report we demonstrate that the closely related molecules IL-17F and IL-17A are secreted as both homodimeric and heterodimeric forms, all of which are biologically active. Activated human CD4+ T cells can secrete all three different molecular structures, confirming that the heterodimer is a natural form of the protein.
IL-17F/A Heterodimer

We hypothesized that IL-17F and IL-17A may be capable of forming heterodimers based upon the fact that the crystal structure of IL-17F shows that the dimeric protein is a cysteine knot family member (23). Cysteine knot family members can exist as heterodimers in vivo (25, 26). To test this hypothesis, we used various in vitro techniques, including ELISAs, immunoprecipitation followed by Western blot, and mass spectrometry. These techniques were validated first using recombinant proteins expressed in COS cells, as shown in Table 1 and Figs. 1–3. The validated assays were then used to show that activated human CD4+ T cells not only secrete IL-17F and IL-17A homodimers but also an IL-17F/IL-17A heterodimer as shown in Table 2 and Figs. 5 and 6.

The mass spectrometric analysis on the conditioned medium from the activated CD4+ T cells (Fig. 6) identified the cysteine containing peptides, which are involved in disulfide bond formation between IL-17F and IL-17A. Of note, the IL-17F cysteines that are involved in forming the heterodimer are the same cysteines utilized in the homodimer formation as seen by mass spectrometry and crystal structure. This suggests that pairing of the heterodimer subunits will occur in a similar manner as that observed in the crystal structure of the IL-17F homodimer. Human IL-17F and IL-17A can signal through a receptor complex composed of the IL-17R and IL-17RC receptor chains (34, 35). The conserved cysteine bond formation in the heterodimer suggests that this protein could signal through the same receptor complex, as the subunits may be predicted to have a conserved orientation. For nerve growth factor, a cysteine knot family member, the interface of the disulfide-linked chains forms a large cavity, and this has been shown to be the high-affinity binding site for the nerve growth factor receptor (36). It is likely that the receptor for IL-17F or IL-17F/IL-17A heterodimers may also bind to the dimer interface.

Of particular interest is our result on the levels of the three cytokines in the conditioned medium of activated CD4+ T cells from six human donors, as determined by ELISA. In all six donors, the IL-17F homodimer was secreted on the order of at least 10-fold higher than the IL-17A homodimer, and in all of the donors the amount of IL-17A homodimer was below the level of detection. This data strongly suggests that the majority of the IL-17A protein expressed by these donors is part of an IL-17F/IL-17A heterodimer. The identification of the heterodimer and the low level of IL-17A homodimer in the six donors leads us to question what the physiological role of these three cytokines might be. In our in vitro functional assays using BEAS-2B cells, all three cytokines induced the secretion of the chemokine GRO-α; however, the IL-17F homodimer was reproducibly 100-fold less active than the IL-17A homodimer (Fig. 4). Similar results were obtained in GRO-α assays using different cell lines (data not shown).

There is increasing evidence indicating a pathogenic role of IL-17A in a wide range of inflammatory and autoimmune diseases. In support of this, recent reports have shown that treatment with an anti-huIL-17A antibody decreased the severity of disease in EAE and CIA mouse models (2, 3, 15). Activated T cells from both mouse and humans are reported to co-express IL-17F and IL-17A (7, 11, 12). However, based on results reported here, it is tempting to propose that the pathogenic activity of IL-17A could be attributed, at least partially, to the IL-17F/IL-17A heterodimeric cytokine. Additional studies should further elucidate the role of these three cytokines in inflammatory diseases and evaluate the therapeutic utility of drugs targeting IL-17 family members.

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