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Tissue and T Cell Distribution of Precursor and Mature IL-16

Geoffrey L. Chupp, Eric A. Wright, David Wu, Margaret Vallen-Mashikian, William W. Cruikshank, David M. Center, Hardy Kornfeld, and Jeffrey S. Berman

IL-16 is a novel cytokine, which is chemoattractant for CD4+ T cells, macrophages, and eosinophils. Recently, it was reported that IL-16 is synthesized as an approximately 80-kDa precursor molecule, pro-IL-16. Since little is known about the processing and tissue distribution of IL-16 and pro-IL-16, we investigated the distribution of IL-16 mRNA and protein in human lymphoid tissue. Northern blotting identified IL-16 mRNA predominantly in normal lymphoid organs, including PBMC, spleen, and thymus. Immunohistochemistry of human lymph node localized IL-16 protein to lymphocyte cytoplasm within T cell zones and occasionally in lymphocytes in B cell zones. Flow cytometric detection of intracellular IL-16 showed that >70% of CD4+ and CD8+ T cells constitutively expressed IL-16 protein. Western blot analysis of PBMC revealed nearly all of this protein to be approximately 80-kDa pro-IL-16 in unstimulated PBMC, and upon cell activation, the amino terminus of pro-IL-16 is processed into multiple fragments. These results show that pro-IL-16 is widely and constitutively expressed and suggest that the amino terminus of the protein can be processed upon cell activation. The Journal of Immunology, 1998, 161: 3114–3119.

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Materials and Methods

Reagents

Recombinant IL-16 was generated as previously described (1). Briefly, a cDNA fragment corresponding to the IL-16 ORF plus nine additional NH4-terminal amino acids (GenBank accession no. M90391, nucleotides 787-1130) was ligated into the Escherichia coli expression vector pET-16b for the generation of a rIL-16-polyspecific fusion protein that was purified by metal chelation chromatography. For the generation of Abs, the fusion
FIGURE 1. Structure and Ab recognition and current understanding of the IL-16 molecule. IL-16 is synthesized as a 631-amino acid precursor (pro-IL-16), and the secreted fragment (IL-16) autoaggregates to form bioactive multimers. mAbs 17.1, 14.1, and Ab 16 detect the secreted portion of IL-16, while Ab 86 detects a 15-amino acid peptide sequence in pro-IL-16.

Antibodies

Rabbit polyclonal anti-pro-IL-16 Ab 86 was prepared by immunizing rabbits with the synthetic peptide 86 (5'-NGKSLKGTTGHDALA-3'). This peptide corresponds to amino acids 465 to 479 of the pro-IL-16 sequence, but is not included in mature IL-16 (Fig. 1). Sequence comparison of this 15-amino acid peptide against GenBank, EMBL, and PIR databases showed no homology with any known protein. Rabbit polyclonal anti-IL-16 Ab 16 was prepared from rIL-16-immunized rabbit sera as previously described (1). Murine anti-IL-16 mAbs 14.1 (isotype IgG2a, κ) and 17.1 (isotype IgG1, κ) were generated by Agmed (New Bedford, MA) and were screened based on their ability to specifically block bioactivity in vitro and recognize rIL-16 by Western blotting. All Abs were purified using protein A as previously described (1). FITC-conjugated mAb 17.1 was prepared using a FluoroReporter kit (Molecular Probes, Eugene, OR) for intracellular cytokine staining. Isotype control mAb were purchased from PharMingen based on work by Sander and colleagues (25, 26). Briefly, 10^6 PBMC were suspended in ﬁltered staining buffer (PBS, 1% heat-inactivated FCS, and 0.1% (w/v) sodium azide, pH 7.4) at 2 x 10^6 cells/ml and labeled with phycoerythrin-conjugated Ab to the desired surface Ag for 30 min. The cells were washed once with staining buffer and centrifuged. The resulting pellet was resuspended in 100 μl of 4% paraformaldehyde and PBS, pH 7.4, for 20 min. After washing with staining buffer and centrifugation, the cells were resuspended in 50 μl of permeabilization buffer consisting of staining buffer and 0.1% saponin (w/v) and incubated with FITC-conjugated anti-IL-16 mAb 17.1 or isotype control for 30 min. The cells were then washed once with permeabilization buffer to remove unbound intracellular Ab, centrifuged, resuspended in 10% buffered formalin, and analyzed by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA). Analysis of 10,000 events within the lymphocyte gate was performed using LYSYS software (Becton Dickinson) and Microsoft Excel (Seattle, WA).

Surface staining control and blocking studies

To control for the possibility of anti-IL-16 mAb binding to the cell surface, cells were suspended as described above in staining buffer, incubated with FITC-conjugated mAb 17.1 or isotype control for 30 min, washed, fixed, and analyzed by FACS. Blocking with rIL-16 was accomplished by pre-incubating FITC-conjugated mAb17.1 or isotype control with 0.3 μg of rIL-16 for 30 min at 4°C. From this solution 0.3 μg of Ab was added to PBMC suspended in permeabilization buffer as described above. After washing with permeabilization buffer, the pellet was resuspended in buffered formalin and analyzed by FACS as described above.

Protein gel electrophoresis and Western blot analysis

Cell preparations as described above were centrifuged at 250 x g, resuspended in 2x SDS sample buffer, boiled for 5 min, and stored at -20°C. Analysis by SDS-PAGE was performed with 3% stacking and 10% polyacrylamide gels according to the method of Laemmli. Western blotting was performed using standard protocols. Detection of specifically bound Ab was performed using a Super Signal detection kit (Pierce), or 125I-labeled protein A.

Stimulation of T cell subsets with immobilized anti-CD3

T-25 tissue culture flasks (Costar) were precoated with anti-CD3 mAb at 1 μg/ml for 2 h at 37°C. After washing with sterile PBS, CD4+ or CD8+ T cells at 2 x 10^6/ml in medium 199/0.4% BSA were plated onto anti-CD3 or uncoated flasks and incubated at 37°C. After 6 and 24 h, cells were harvested and centrifuged, and the cell pellets were diluted in 2x SDS sample buffer.

Histological procedures and found to be normal reactive lymphoid tissue were

immunohistochemistry

Two human lymph nodes acquired from excess tissue resected during surgical procedures and found to be normal reactive lymphoid tissue were embedded in optimal cutting temperature compound and snap-frozen at -70°C. Sections were fixed in cold methanol for 5 min and then washed with PBS. Immunohistochemistry was performed with a Vectastain ABC kit according to the manufacturer's protocol (Vector Laboratories, Burlingame, CA). mAb 14.1 or isotype control were diluted in PBS, pipetted onto sections, and incubated overnight at 4°C. Secondary Abs were labeled with biotin, and detection was performed with a strepavidin-horseradish peroxidase conjugate and diaminobenzidine substrate. Slides were counterstained with hematoxylin, dehydrated in graded ethanol solutions, equilibrated with xylene, and coverslipped. Representative sections were photographed with Kodak Ektachrome 60T (Eastman Kodak, Rochester, NY). Images were prepared with Adobe Photoshop (Mountain View, CA).
Results

IL-16 expression in multiple tissues

Tissue expression of IL-16 mRNA was evaluated by Northern blot analysis of poly(A)^+ RNA extracted from a variety of human organs and hybridized with a ^32P-labeled fragment of the human IL-16 cDNA (Fig. 2). A single band of approximately 2.8 kb was observed in lanes containing RNA from spleen, thymus, and peripheral blood leukocytes. Little or no signal was observed from prostate, testis, or colon after prolonged autoradiograph exposure. In a separate Northern blot (not shown), a strong band at 2.8 kb was observed in poly(A)^+ RNA from pancreas, and very weak signals were observed in placenta, lung, liver, and brain. Thus, in the absence of disease, IL-16 mRNA expression was mainly restricted to the lymphoid compartment.

To determine whether IL-16 protein was detectable in peripheral lymphoid tissue, mAb 14.1 against IL-16 (see Fig. 1) was used for immunohistochemical staining of two human lymph nodes. A representative section is presented in Figure 3. In all specimens examined, IL-16 staining was observed in the majority of the cells in the mantle (T cell zone); staining of lymphocyte cytoplasm and lack of nuclear staining suggested that IL-16 was being identified in the cell cytoplasm. Conversely, relatively few cells expressed IL-16 within follicles (B cell zone). No signal was detectable in sections stained with isotype control mAb. These data corroborated the Northern blot findings and showed that IL-16 protein was expressed in the majority of lymphocytes in the T cell zones in lymph node.

Detection of IL-16 in unstimulated CD4^+ and CD8^+ T lymphocytes

Because CD8^+ T cells were previously found to constitutively express IL-16 bioactivity (22), we first characterized the expression of IL-16 protein in CD8^+ T cells. To accomplish this we used FITC-conjugated anti-IL-16 mAb 17.1, which recognizes an epitope on the C-terminal of the IL-16 molecule (see Fig. 1). A protocol employing saponin treatment was used to detect both surface and intracellular proteins. Two-color flow cytometry for CD8 vs IL-16 of unstimulated PBMC within the lymphocyte gate showed that 75% of CD8^+ T cells expressed IL-16. D, Staining for IL-16 vs CD8 in the absence of saponin shows that no IL-16 is expressed on the cell surface of CD8^+ or CD8^- T cells within the lymphocyte gate. E and F, Preincubation of anti-IL-16 mAb with rIL-16 completely blocked the detection of intracellular IL-16 in both CD4^+ (E) and CD8^+ (F) T lymphocytes.
IL-16 (Fig. 4C). This finding was unexpected since CD4+ T cells have not been shown to contain preformed bioactive protein. Similar percentages of CD8+ (86 ± 8.98%) and CD4+ (83 ± 8.18%) T cells stained for IL-16 in subsequent experiments using PBMC from different donors.

To confirm that mAb 17.1 was detecting intracellular protein and not binding to the cell surface, unstimulated PBMC were stained with FITC-conjugated mAb 17.1 in standard staining buffer in the absence of saponin. There was no detectable IL-16 on the surface of CD8+ or CD8- lymphocytes, confirming that mAb 17.1 was detecting intracellular IL-16 (Fig. 4D). The specificity of this signal for IL-16 was confirmed by preincubating FITC-conjugated mAb 17.1 with rIL-16, which completely blocked the detection of intracellular IL-16 in both CD4+ and CD8- lymphocytes (Fig. 4, E and F). These data indicated that the majority of resting unstimulated CD4+ and CD8+ T cells express IL-16 and that the antigenic portion of IL-16 resides in the intracellular compartment.

Identification of pro-IL-16 in unstimulated PBMC

To confirm the flow cytometry results, duplicate Western blots of rIL-16 and PBMC lysates were probed with the three anti-IL-16 Abs (Ab 16, and mAbs 14.1 and 17.1; see Fig. 1) and control Abs (Fig. 5A). Western blotting with secondary alone or control Ab detected no protein (not shown); however, probing with mAbs 17.1, 14.1, and Ab 16 recognized both 17-kDa rIL-16 and a prominent pro-IL-16 band at about 80 kDa. A third weak band migrating at about 60 kDa was detected when more protein was run per lane or with longer film exposure (not shown). The abundance of pro-IL-16 was evident, since the approximately 80-kDa band was prominent in lysate prepared from as few as 2.5 × 10^6 PBMC.

To verify that the approximately 80-kDa band recognized by Abs raised to rIL-16 was pro-IL-16, Ab 86 against pro-IL-16 (see Fig. 1) was used to probe a Western blot of rIL-16, PBMC lysate, and in vitro translated pro-IL-16 (Fig. 5A, blot 4). This Ab identified the approximately 80-kDa band in unstimulated PBMC with approximately equal intensity to that seen with Abs against IL-16, but did not recognize rIL-16.

To substantiate that the approximately 80-kDa band detected by mAb 17.1 was pro-IL-16, we probed a Western blot of rIL-16, unstimulated PBMC and in vitro translated pro-IL-16 with mAb 17.1 and identified comigrating bands in both the PBMC and in vitro translated pro-IL-16 lanes (Fig. 5B). These data validated that mAbs against rIL-16 recognize native pro-IL-16 and show that pro-IL-16 is abundant in CD4+ and CD8+ T cells, and that the majority of IL-16 protein identified by flow cytometry is in the form of pro-IL-16.

In contrast to the abundant presence of pro-IL-16, IL-16 was not abundant in unstimulated PBMC and was only intermittently detectable on immunoblots of cell lysates. Figure 5C is a representative Western blot using Ab 16 as a primary Ab and [125I]protein A for detection of PBMC before and after magnetic bead depletion of CD4+ T cells. In this donor, pro-IL-16 and the mature form of IL-16 were detectable in NWNT before depletion (lane d) and in purified CD8+ T cells after magnetic bead depletion (lane e). The 121-amino acid bioactive form of IL-16 has not been detectable by Western blot in purified CD4+ T cells (not shown).

Processing of pro-IL-16 in CD4+ and CD8+ T cells after anti-CD3 stimulation

The data presented show that pro-IL-16 is abundant and constitutively expressed in CD4+ and CD8+ T cells. We hypothesized that upon stimulation with immobilized anti-CD3, pro-IL-16 would be processed releasing IL-16. Purified CD4+ or CD8+ T cells were incubated with plate-bound anti-CD3 and harvested after 6 and 24 h (Fig. 6). Western blotting of harvested cell lysates revealed a prominent pro-IL-16 band at about 80 kDa of equal intensity in unstimulated CD4+ and CD8+ T cell lysates (lanes a and b, respectively). After 6 h the pro-IL-16 band was preserved, and several lower m.w. bands appeared in both CD4+ (lane c) and CD8+ (lane d) T cells, which at 24 h were less prominent (lanes e, CD4+ at 24 h; lane f, CD8+ at 24 h). These data suggested that pro-IL-16 may be processed upon cell stimulation. The hypothesis that mature IL-16 is released by processing of pro-IL-16 is further supported by the observation that native secreted IL-16 is weakly, if at all, detectable in low amounts on Western blots of cell lysates with the Abs used in these experiments (for example, Fig. 5C). Although we did find IL-16 chemoattractant bioactivity in culture supernatants that was specifically inhibited by anti-IL-16 Abs, there was insufficient IL-16 protein in the supernatants to detect an
approximately 17-kDa band consistent with IL-16 in these experiments (not shown).

**Discussion**

IL-16 is a novel cytokine bearing no homology to other proteins possessing lymphocyte chemoattractant activity (27). Specifically, IL-16 contains a single cysteine at position 22, the IL-16 gene is not located on the chemokine cluster, and it lacks a signal peptide typical of proteins secreted via the Golgi apparatus. The recent identification of pro-IL-16, however, suggests that IL-16 has similarities without structural homology to IL-1, being proteolytically cleaved by an IL-converting enzyme-like protein upon cell activation (20, 28). The current investigations show that pro-IL-16 is constitutively distributed in abundance in circulating human T cells, spleen, and thymus, and suggest that pro-IL-16 is proteolytically processed at several sites upon cell activation.

The initial experiments identifying IL-16 mRNA primarily in lymphoid organs (Fig. 1) suggest that under normal conditions IL-16 may participate in immune system homeostasis in nondiseased states. This hypothesis is further supported by the finding of widespread expression of IL-16 protein in the T cell zone in human lymphoid tissue (Fig. 2). It is also likely that IL-16 has alternative functions in pathologic states (29), where IL-16 expression is not restricted to lymphoid cells. For example, in asthmatic lung, in functions in pathologic states (29), where IL-16 expression is not widespread expression of IL-16 protein in the T cell zone in human lymphoid organs (Fig. 1) suggest that under normal conditions IL-16 may participate in immune system homeostasis in nondiseased states. This hypothesis is further supported by the finding of widespread expression of IL-16 protein in the T cell zone in human lymphoid tissue (Fig. 2). It is also likely that IL-16 has alternative functions in pathologic states (29), where IL-16 expression is not restricted to lymphoid cells. For example, in asthmatic lung, T cell accumulation, the bronchial epithelium can be an alternate source of IL-16 (14).

The experiments aimed at the FACS detection of IL-16 in unstimulated PBMC were based on previous data showing IL-16 bioactivity in unstimulated CD8+ T cell lysates (22). We hypothesized that flow cytometry would detect IL-16 in a subset of unstimulated CD8+ T cells, but found that nearly all unstimulated CD8+ T cells stained for IL-16 (Fig. 4). Further, we detected IL-16 in nearly all unstimulated CD4+ T cells. This was unexpected, as CD4+ T cell lysates have not been shown to contain IL-16 chemoattractant activity, and required that we confirm that the abundant protein in CD4+ and CD8+ T cells detected by flow cytometry signal was IL-16. Western blotting with three Abs against secreted IL-16 peptide and one Ab against pro-IL-16 showed that the abundant protein identified by flow cytometry was the approximately 80-kDa pro-IL-16 (Fig. 5. A and B).

Taken together with the fact that CD4+ T cells express IL-16 mRNA (22) and that IL-16 chemoattractant activity is detectable in supernatants of CD4+ T cell cultures after 6 h of stimulation with immobilized anti-CD3 (D. Wu, manuscript in preparation), these data suggest that the processing of pro-IL-16 leading to the secretion of mature IL-16 differs between CD8+ and CD4+ T cells. While both cell types constitutively express pro-IL-16, however, CD8+ T cells also constitutively express a significant pool of mature IL-16, while CD4+ T cells constitutively express little or no mature IL-16, requiring cell activation to secrete mature IL-16.

Western blotting also suggested that upon stimulation with Ag, pro-IL-16 is cleaved at alternative sites (Fig. 6). Since the Ab used for Western blotting in this experiment detects the carboxyl terminus of IL-16, these data suggest that the amino terminus of pro-IL-16 is processed. Recently, caspase-3, an IL-1-converting enzyme family member, has been shown in vitro to cleave a 50-kDa pro-IL-16 peptide releasing an approximately 20-kDa fragment possessing IL-16 bioactivity (30). Our studies suggest that other IL-16 cleavage sites are also likely to exist (Fig. 5C). The physiologic relevance of these cleavage fragments to IL-16 function or degradation is not yet known. We have also noted that when protein is extracted from resting T cells with nonionic detergent rather than SDS, similar bands are detectable. Although it is possible that pro-IL-16 is susceptible to degradation by nonionic detergent, a more plausible explanation is that during nonionic detergent solubilization, pro-IL-16 processing enzymes resistant to standard protease inhibitors (but inactivated by SDS) may be able to cleave pro-IL-16. This explanation is further supported by the finding that similar bands were detected by Western blot analysis of anti-CD3-stimulated T cell lysates extracted in SDS sample buffer (Fig. 6) and may explain why we detect a predominant approximately 80-kDa band in unstimulated PBMC rather than processed fragments identified by others (20).

To determine the effect of cell activation on pro-IL-16 expression, we stimulated T cells with immobilized anti-CD3. Since previous studies showed that release of bioactive IL-16 from serotonin-stimulated CD8+ T cells does not require new protein synthesis (23), we hypothesized that TCR ligation would result in the processing of pro-IL-16 and the release of mature IL-16. We were unable to detect mature IL-16 in stimulated cell lysates, but found that the amino terminus of pro-IL-16 was cleaved upon cell activation (Fig. 6). The significance of these activation-induced cleavage products is unknown. Pro-IL-16 also remained abundant following cell stimulation, suggesting that only a small proportion of pro-IL-16 is processed at the carboxyl terminus to release mature IL-16 and, since native mature IL-16 is weakly, if at all, detectable in low amounts on Western blots with the Abs used in these experiments (Fig. 5C), that the preformed pool of pro-IL-16 is large compared with the amount of processed secreted IL-16.

It is unlikely that pro-IL-16 has bioactivity ascribed to IL-16, such as chemoattractant activity or inhibition of HIV replication. Pro-IL-16 is abundant in peripheral blood CD4+ T cells, yet there is a lack of chemoattractant activity in unstimulated CD4+ T cell lysates (22), and peripheral blood CD4+ T cells support HIV replication.

This study shows that pro-IL-16 is a substantial component of the majority of unstimulated blood T cells. The pathway of IL-16 synthesis and secretion remains largely obscure and continues to be actively investigated. It is not known whether mature IL-16 can
be secreted by a subset of lymphocytes capable of cleaving pro-IL-16 or if all pro-IL-16-expressing cells have the capacity to secrete mature IL-16. It is also unknown whether the amino terminal processed products of cell activation represent intermediate forms of IL-16 before the release of mature IL-16, or if these proteins represent processing products with alternative intra- or extracellular functions. Further investigations will be required to detect other functional roles for this protein.

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