Autocrine/paracrine cytokine stimulation of leukemic cell proliferation in smoldering and chronic adult T-cell leukemia

Jing Chen,1 Mike Petrus,1 Bonita R. Bryant,1 Vinh Phuc Nguyen,1 Carolyn K. Goldman,1 Richard Bamford,2 John C. Morris,1 John E. Janik,1 and Thomas A. Waldmann1

1Metabolism Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD; and 2Transponics, Jacobus, PA

Introduction

Adult T-cell leukemia (ATL), a heterogeneous disease, can be divided into smoldering, chronic, lymphoma, and acute types clinically. In addition to different clinical manifestations, different stages of ATL have different molecular signatures. Here, we demonstrated that smoldering/chronic ATL peripheral blood mononuclear cells spontaneously proliferated ex vivo in a cytokine (interleukin-12 [IL-12]/IL-9/IL-15)–dependent manner, while acute-type ATL peripheral blood mononuclear cells did not proliferate or proliferated independent of cytokines. Smoldering/chronic ATL cells produced IL-2 and IL-9 in 6-day ex vivo cultures. Interestingly, the addition of an anti-IL-2R-α monoclonal antibody profoundly inhibited IL-9 expression, suggesting optimal expression of IL-9 was dependent on IL-2 signaling in these patients. To determine whether there would be autonomous proliferation of ATL leukemic cells, we purified leukemic cells from patients with smoldering/chronic ATL. Purified leukemic cells cultured alone produced IL-2/IL-9, and the downstream Janus kinase/signal transducer and activator of transcription pathway was activated. However, the leukemic cells did not proliferate independently, but required coculture with autologous monocytes to induce proliferation. Moreover, interaction between leukemic cells and monocytes was contact dependent, and major histocompatibility complex class II expression may have contributed to this interaction. In conclusion, our data provide evidence that there is autocrine/paracrine cytokine stimulation of leukemic cell proliferation in patients with smoldering/chronic ATL that could be targeted for treatment.

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Cell culture, antibodies, and reagents

All cells were cultured in RPMI 1640 plus 10% fetal bovine serum (FBS). NK-92, a cytokine-dependent natural killer (NK) cell line, was maintained with the addition of 60 U/mL of recombinant human IL-2 (rhIL-2). The IL-2– and IL-15–neutralizing monoclonal antibodies were obtained from R&D Systems, and the IL-9– and IL-9R–neutralizing antibodies were purchased from Biologend. Daclizumab (humanized anti–IL-2R-α) was purchased from Hoffmann-La Roche. HuMik-β1 (humanized anti–IL-2/IL-15R-β) was produced by the Biopharmaceutical Development Program of the NCI.

Six-day spontaneous proliferation assay

Human PBMCs were isolated by Ficoll-Hypaque density centrifugation. The PBMCs were washed with phosphate-buffered saline (PBS) and then resuspended in RPMI 1640 media supplemented with 10% FBS at a concentration 1 × 10^6 cells/mL. Then, 100-μL aliquots of the cell suspensions were seeded into 96-well microtiter plates in duplicates and cultured for 6 days at 37°C in 5% CO₂. Antibodies were added at the initiation of the culture period at 10 μg/mL. If 2 antibodies were combined, the concentration of each antibody was 5 μg/mL. Cultures were pulsed with 1 μCi ^3H thymidine 6 hours before harvesting.

Taqman real-time quantitative RT-PCR

Total RNA was extracted from PBMCs using the RNasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Reverse transcription (RT) reactions were carried out for each sample (250 ng) using the Superscript First-Strand Synthesis System (Invitrogen). The Taqman Universal PCR Master Mix, the human IL-2, IL-9 primer/probe, HTLV-I Tax primer/probe, and the HPRT1 primer/probe sets were purchased from Applied Biosystems. The HTLV-I Tax primer/probe has been described previously. The detection of human IL-2, IL-9, HTLV-I Tax, and HPRT1 was performed using an ABI prism 7500 sequence detection system (Applied Biosystems) according to the manufacturer’s instructions. The copy numbers of IL-2, IL-9 mRNA, and HTLV-I Tax mRNA were normalized to the copy number of human HPRT1 mRNA.

IL-2, IL-15, and IL-9 ELISA

IL-2 and IL-15 were measured using the human IL-2 and IL-15 quantikine enzyme-linked immunosorbent assay (ELISA) kit (D2050/D1500; R&D Systems). IL-9 was measured using the LEGEND MAX Human IL-9 ELISA kit (BioLegend).

NK-92 proliferation assay

 Supernatants from ATL PBMC cultures were collected at day 6. NK-92 cells were washed 4 times in PBS, then resuspended in RPMI 1640 media plus 10% FBS and kept at 37°C to release surface-bound IL-2. Four hours later, the cells were washed 4× in PBS, then resuspended in RPMI 1640 media plus 10% FBS and starved overnight at 37°C in 5% CO₂. The rest NK-92 cells were then cultured with ATL PBMC culture supernatants for 48 hours. Antibodies were added at the initiation of the culture period at 10 μg/mL. Cultures were pulsed with 1 μCi ^3H-thymidine 6 hours before harvesting.

ATL leukemic cell and monocyte separation

ATL leukemic cell and monocyte separations were performed using the human regulatory T-cell (Treg) separation kit and human monocyte isolation kit II (Miltenyi Biotec) respectively. ATL leukemic cells were separated by a 2-step purification procedure. First, non-CD4⁺ cells were labeled by a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR-γδ, and CD235a. After incubation with anti-biotin microbeads, the non-CD4⁺ cells were depleted by separation over a MACS column. In the second step, CD4⁺ CD25^high cells were directly labeled with CD25 MicroBeads and isolated by positive

Methods

Patient materials

All patient samples were obtained from patients under the care of the clinical trials team of Metabolism Branch, National Cancer Institute (NCI). ATL subtypes classified according to the diagnostic criteria proposed by Shimoyama et al were: (1) smoldering type: 5% or more abnormal lymphocytes of T-cell nature in peripheral blood, normal lymphocyte levels (< 4 × 10⁹/L), no hypercalcemia (corrected calcium level < 2.74mM), lactate dehydrogenase (LDH) value of up to 1.5 times the normal upper limit; (2) chronic type: absolute lymphocytosis (4 × 10⁹/L or more) with T lymphocytes more than 3.5 × 10⁹/L, LDH value up to twice the normal upper limit, no hypercalcemia; (3) lymphoma type: no lymphocytosis, 1% or less circulating abnormal T lymphocytes, and histologically proven lymphadenopathy with or without extranodal lesion; and (4) acute type: remaining ATL patients who have usual leukemic manifestations and tumor lesions. The clonality of ATL cells was analyzed by polymerase chain reaction (PCR) to look at T-cell receptor (TCR) rearrangement in the tumor lesions. The clonality of ATL cells was analyzed by polymerase chain reaction (PCR) to look at T-cell receptor (TCR) rearrangement in the tumor lesions. The clonality of ATL cells was analyzed by polymerase chain reaction (PCR) to look at T-cell receptor (TCR) rearrangement in the tumor lesions. The clonality of ATL cells was analyzed by polymerase chain reaction (PCR) to look at T-cell receptor (TCR) rearrangement in the tumor lesions. The clonality of ATL cells was analyzed by polymerase chain reaction (PCR) to look at T-cell receptor (TCR) rearrangement in the tumor lesions. The clonality of ATL cells was analyzed by polymerase chain reaction (PCR) to look at T-cell receptor (TCR) rearrangement in the tumor lesions.
selection from the pre-enriched CD4+ T-cell fraction. All the cell separations were performed using an AutoMACS cell separator (Miltenyi Biotec) according to the manufacturer’s instruction.

FACS and intracellular staining

anti-CD4FITC, anti-CD25PE, anti-CD3APC, and isotype controls were purchased from BD Biosciences. Phospho-STAT5 Alexa 488 was obtained from Cell Signaling Technology. To stain cell-surface markers, the cells were blocked by a human FcR binding inhibitor (eBioscience) at 4°C for 20 minutes, the cells were then stained with the fluorescence-activated cell sorting (FACS) developing antibody at 4°C for 30 minutes. After the wash step, the cells were analyzed using a FACSCaliber (BD Biosciences). The intracellular staining of p-STAT5 followed the method of Ilangumaran et al.19

Results

Cytokine-dependent spontaneous proliferation of smoldering/chronic ATL PBMCs in 6-day ex vivo culture

Adult T-cell leukemia can be divided into smoldering, chronic, lymphoma type, and acute type clinically. We analyzed the spontaneous proliferation of PBMCs from 20 patients with ATL without the addition of mitogens or antigens and compared their proliferation with that of normal healthy donors (n = 20). Among the 20 patients with ATL, 11 patients had smoldering/chronic type ATL (9 smoldering ATL, 2 chronic ATL) and 9 patients had acute-type ATL. The 11 patients with smoldering/chronic ATL studied were in the very early phase of their leukemia, as only 6 patients showed monoclonal leukemic population by PCR. PBMCs from all patients with smoldering/chronic ATL proliferated ex vivo in RPMI 1640 media/10% FBS without the addition of any antibody (3000 cpm; mean, 45 593 cpm) (Figure 1A). In contrast, the PBMCs from most of the patients with acute-type ATL did not proliferate (3000 cpm; 7 of 9 patients), with the exception of 2 patients whose PBMCs proliferated at 23 262 and 28 569 cpm, respectively. PBMCs from normal donors did not proliferate ex vivo (< 3000 cpm; n = 20). Furthermore, the proliferation of PBMCs from smoldering/chronic-type ATL was profoundly inhibited by the addition of antibodies to IL-2 or IL-2R-α, IL-15 and IL-2/15R-β, IL-9 and IL-9R-α, and of a nonspecific control antibody (UPC10). (C) The spontaneous proliferation of PBMCs from a patient (patient 4) with smoldering/chronic ATL was assayed in the presence of 10 μg/mL monoclonal antibodies to IL-2, IL-9, IL-15, the combinations of anti-IL-2 with anti-IL-15, anti-IL-2 and anti-IL-9, and with a nonspecific control antibody (UPC10). This represents the data from 3 patients. (D) Spontaneous proliferation of PBMCs from 2 patients with acute ATL in the presence of 10 μg/mL monoclonal antibodies to IL-2 and IL-2R-α, IL-15 and IL-2/15R-β, IL-9 and IL-9R-α, and of a nonspecific control antibody (UPC10). The PBMCs proliferated at 23 262 and 28 569 cpm, respectively, without the addition of any antibody. The percentage inhibition was calculated by comparing the proliferation to that without any antibody.

Figure 1. PBMCs from patients with smoldering/chronic ATL manifested autocrine/paracrine cytokine-dependent spontaneous proliferation. (A) The 6-day spontaneous proliferation of ex vivo PBMCs was assayed on cells from smoldering/chronic ATL (n = 11, 9 smoldering ATL, 2 chronic ATL), acute-type ATL (n = 9), and normal donors (n = 20). (B) Spontaneous proliferation of smoldering/chronic ATL PBMCs (n = 11) was assayed in the presence of 10 μg/mL of monoclonal antibodies to IL-2 and IL-2R-α, IL-15 and IL-2/15R-β, IL-9 and IL-9R-α, and of a nonspecific control antibody (UPC10). (C) The spontaneous proliferation of PBMCs from a patient (patient 4) with smoldering/chronic ATL was assayed in the presence of 10 μg/mL monoclonal antibodies to IL-2, IL-9, IL-15, the combinations of anti-IL-2 with anti-IL-15, anti-IL-2 and anti-IL-9, and with a nonspecific control antibody (UPC10). This represents the data from 3 patients. (D) Spontaneous proliferation of PBMCs from 2 patients with acute ATL in the presence of 10 μg/mL monoclonal antibodies to IL-2 and IL-2R-α, IL-15 and IL-2/15R-β, IL-9 and IL-9R-α, and of a nonspecific control antibody (UPC10). The PBMCs proliferated at 23 262 and 28 569 cpm, respectively, without the addition of any antibody. The percentage inhibition was calculated by comparing the proliferation to that without any antibody.
Supernatants. As shown in Table 1, the smoldering/chronic ATL PBMCs secreted meaningful quantities of IL-2 and IL-9 into the ex vivo culture supernatants, whereas IL-2 and IL-9 could not be detected in the 6-day culture supernatants of normal donors (data not shown). IL-15 was not detected in the ex vivo culture supernatants of ATL cells, as assessed both by ELISA and by NK-92 stimulation and proliferation.

IL-2 was required for IL-9 expression in smoldering/chronic ATL PBMCs

To better understand the kinetics of cytokine production in the ex vivo cultures of PBMCs from patients with smoldering/chronic ATL, we used Taqman real-time RT-PCR to detect the production of message for IL-2, IL-9, and Tax at different time points during the 6-day culture. As shown in Figure 3A, Tax mRNA expression was not detectable at day 0, but was induced at day 1 and then stabilized from days 2 through 6. The IL-2 mRNA expression was dramatically induced at day 1 and then decreased during the culture period. Interestingly, IL-9 message was not induced until day 2 and increased over the culture period (Figure 3A). This delayed expression of IL-9 prompted us to examine the relationship between IL-2 and IL-9 expression. To determine whether IL-2 signaling would be needed for IL-9 production, we added a monoclonal antibody to IL-2R-α at the initiation of the ATL PBMC culture period and analyzed the cells for IL-9 message expression at subsequent time points. As shown in Figure 3B, the IL-9 expression by smoldering/chronic ATL PBMCs was dramatically reduced by the addition of the anti–IL-2R-α antibody, while the expression of IL-2 and Tax was not changed by the addition of anti–IL-2R-α. ELISA analysis for IL-2 and IL-9 in the supernatants confirmed the decrease in IL-9 protein production that was associated with the addition of an anti–IL-2R-α antibody (Table 2).

Table 1. IL-2 and IL-9 protein was secreted in most of the PBMC cultures of patients with smoldering/chronic ATL

<table>
<thead>
<tr>
<th>Patient no</th>
<th>IL-2 (pg/mL)</th>
<th>IL-9 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATL1</td>
<td>Not detected*</td>
<td>9975</td>
</tr>
<tr>
<td>ATL2</td>
<td>10</td>
<td>296</td>
</tr>
<tr>
<td>ATL3</td>
<td>391</td>
<td>1374</td>
</tr>
<tr>
<td>ATL4</td>
<td>1196</td>
<td>532</td>
</tr>
<tr>
<td>ATL5</td>
<td>358</td>
<td>161</td>
</tr>
<tr>
<td>ATL6</td>
<td>Not detected*</td>
<td>310</td>
</tr>
<tr>
<td>ATL7</td>
<td>11</td>
<td>359</td>
</tr>
<tr>
<td>ATL8</td>
<td>1639</td>
<td>945</td>
</tr>
<tr>
<td>ATL9</td>
<td>47</td>
<td>Not done</td>
</tr>
<tr>
<td>ATL10</td>
<td>Not detected*</td>
<td>2214</td>
</tr>
<tr>
<td>ATL11</td>
<td>Not detected*</td>
<td>10</td>
</tr>
</tbody>
</table>

*The level of IL-2 was < 7 pg/mL.
chronic ATL and examined the IL-2 production by the purified leukemic cells. ATL leukemic cells are usually phenotypically characterized as CD4+CD25brightCD3dim cells. We selected for leukemic cells based on their surface expression of CD4 and their high level of expression of CD25. FACS analysis of these purified leukemic cells confirmed that they also express decreased levels of CD3, compared with the nonleukemic CD3-expressing population (Figure 4A). The purity of the selected leukemic cell population was approximately 92%-97% (Figure 4A). When placed into culture, the purified leukemic cells alone expressed the message for IL-2 at day 2, as assessed by Taqman analysis (Figure 4B).

Furthermore, the supernatants of the purified leukemic cell cultures contained 598 pg/mL of IL-2 protein (Figure 4B). To investigate whether IL-2 activates downstream signaling pathways in purified leukemic cells, since they all expressed high levels of IL-2Rα (CD25) on their surface, we analyzed the activation status of STAT5. As shown in Figure 4C, STAT5 was phosphorylated in purified leukemic cells cultured alone for 2 days. This suggested that ATL leukemic cells were able to make IL-2 in the ex vivo cultures, and that the cytokine could signal through the high-affinity IL-2 receptor on the cell surfaces of the leukemic cells.

Monocytes were required to initiate the spontaneous proliferation of purified ATL leukemic cells

We next examined whether the purified ATL leukemic cells could proliferate spontaneously ex vivo. Surprisingly, the purified ATL leukemic cells, when cultured alone, did not proliferate, although they produced IL-2 and activated the Jak-STAT pathway in the ex vivo cultures (Figure 5A). Previously, we showed that purified ATL T cells proliferated when cocultured with autologous monocytes. We then tested, in the present study, if coculture with autologous monocytes could induce the proliferation of purified ATL leukemic cells. When cultured with autologous monocytes, the purified ATL leukemic cells proliferated, and this proliferation was partially inhibited by the addition of an antibody to IL-2 (Figure 5A). These data suggested that although autocrine IL-2 stimulation was important for the spontaneous proliferation of ATL leukemic cells, that it was not sufficient to induce the proliferation of ATL leukemic cells. Stimulation from autologous monocytes was also required for proliferation. More interestingly, when we purified CD4+CD25brightCD3dim leukemic cells from 2-day ATL PBMC cultures, the purified leukemic cells alone proliferated ex vivo (Figure 5B). This proliferation was also partially inhibited by the

Table 2. The production of IL-9 was decreased by a monoclonal anti–IL-2Rα antibody in ex vivo cultures of PBMCs from a patient with smoldering/chronic ATL

<table>
<thead>
<tr>
<th>PBMC culture</th>
<th>IL-2 (pg/mL)</th>
<th>IL-9 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without anti–IL-2Rα</td>
<td>With anti–IL-2Rα</td>
</tr>
<tr>
<td>Day 1</td>
<td>585</td>
<td>528</td>
</tr>
<tr>
<td>Day 2</td>
<td>954</td>
<td>957</td>
</tr>
<tr>
<td>Day 3</td>
<td>1048</td>
<td>1170</td>
</tr>
<tr>
<td>Day 4</td>
<td>1138</td>
<td>1424</td>
</tr>
<tr>
<td>Day 5</td>
<td>1156</td>
<td>1633</td>
</tr>
<tr>
<td>Day 6</td>
<td>1196</td>
<td>1704</td>
</tr>
</tbody>
</table>

*The level of IL-9 was < 1 pg/mL.
addition of an antibody to IL-2 (Figure 5B). These data suggested that IL-2 signaling was required throughout the ex vivo culture, whereas monocytes were required to be present only early in the ex vivo cultures to initiate the proliferation.

MHC class II expression contributed to the contact-dependent interaction between leukemic cells and monocytes

To better understand the interaction between ATL leukemic cells and autologous monocytes, we performed a 2-chamber T-cell proliferation assay. When the purified leukemic cells and purified monocytes were put into different chambers of the Transwell, which allows only soluble factors to pass through, the purified leukemic cells did not proliferate (Figure 6A). When the purified leukemic cells and purified monocytes were put into the same chamber, proliferation was induced (Figure 6A). This suggested that the contact between leukemic cells and monocytes was important for proliferation, whereas soluble factors made by monocytes were not sufficient. To further examine the latter point, we cultured purified leukemic cells with exogenously added recombinant cytokines that could be produced by monocytes (IL-1α, IL-1β, IL-6, and IL-12) and by leukemic cells (IL-2, IL-9).

None of the exogenously added cytokines was able to induce the purified leukemic cells to proliferate (Figure 6B). Monocytes from ATL patients expressed high levels of major histocompatibility complex (MHC) class I and class II molecules. We then determined if the MHC molecules were involved in the interaction...
between leukemic cells and monocytes. The addition of a monoclonal antibody to MHC class II partially inhibited the spontaneous proliferation induced by the coculture of leukemic cells and monocytes (Figure 6C). Moreover, an anti–MHC class II antibody also partially inhibited the proliferation of smoldering/chronic ATL PBMC cultures (Figure 6D). These results suggest that MHC molecules might contribute to the contact-dependent interaction between leukemic cells and monocytes.

**Discussion**

ATL can be divided into smoldering, chronic, lymphoma, and acute types, based on their clinical manifestations. Other than clinical symptoms, different stages of ATL have different molecular signatures. Although the mechanisms underlying the progression from smoldering/chronic to the acute stage are unknown, various genetic events as well as epigenetic events involved in the progression have been reported. These include the accumulation of gene mutations that break down cell-cycle regulation (eg, p53 and p16 mutations), down-regulation or silencing of negative regulators, such as gene down-regulated by V-Src, Src homology 2 domain–containing protein tyrosine phosphatase-1, and ASY/Nogo, expression of acute-phase–specific genes that are associated with cell growth and apoptosis resistance (eg, MET, NCOA3, HSPD1, GTF3A, and BIRC5), expression-pattern change of HTLV-1 Tax, increasing genomic instability and chromosome abnormalities, and epigenetic modifications including DNA hypermethylation in specific target genes and the presence of CIMP (CpG island methylator phenotype) that involves multigene methylation simultaneously.

In this study, our data clearly demonstrated the cytokine (IL-2, IL-9, and IL-15)–dependent spontaneous proliferation in smoldering/chronic ATL PBMCs as an addition of monoclonal antibodies to cytokines or cytokine receptors inhibited the ex vivo spontaneous proliferations (anti–IL-2/anti–IL-2Rα (CD25), 76%/80%; anti–IL-15/anti–IL-2/15Rβ (CD122), 37%/43%; anti–IL-9/anti–IL-9Rα (CD129), 41%/56% (Figure 1B). Furthermore, the smoldering/chronic PBMCs expressed IL-2 and IL-9 message, as assessed by Taqman analysis (Figure 3), and the supernatants from PBMC cultures from patients with smoldering/chronic ATL contained IL-2 and IL-9 (Tables 1-2). IL-15 was not detected in any of the 11 smoldering/chronic ATL supernatants, possibly due to high-affinity binding of IL-15 to its receptor on cell surfaces. The 11 smoldering/chronic ATL patients studied appeared to be in the very early phase of their leukemia, as only 6 patients showed monoclonal leukemic population by PCR at the time the ex vivo proliferation was performed. On the contrary, acute-type ATL either did not spontaneously proliferate (no proliferation in 7 of 9 cases; Figure 1A) or only proliferated independent of autologous cytokines (IL-2/IL-9/IL-15; Figure 1D). They did not generate and secrete IL-2 and IL-9 into the ex vivo cultures either (data not shown). This suggested that they had gone past the cytokine (IL-2/IL-9/IL-15)–dependent phase of the disease. Consistent with this observation, in our phase 2 trial with daclizumab (anti–IL-2Rα), we observed partial or complete remissions in some patients with smoldering/chronic ATL, but this antibody appears to have little activity in the more aggressive acute subtype of ATL (J. Berkowitz, T.A.W., and J.C.M., unpublished data, October 2010).

To further determine whether there is an autocrine IL-2–mediated stimulation in the ATL leukemic cells, we purified ATL leukemic cells from the PBMCs of patients with smoldering/
chronic ATL. The purified ATL leukemic cells alone produced IL-2 (Figure 4B) and IL-9 (data not shown) in ex vivo cultures and activated the Jak/STAT-signaling pathway. Because ATL leukemic cells express high levels of CD25, the IL-2 receptor alpha, but not the IL-9 receptor alpha, the activation of STAT5 was probably due to IL-2 signaling. Despite the expression of IL-2/IL-2Rα and the activation of the Jak/STAT pathway, the purified ATL leukemic cells alone did not proliferate spontaneously (Figure 5A). They needed help from autologous monocytes, as the coculture of purified leukemic cells with purified monocytes induced proliferation (Figure 5A). Interestingly, the monocytes appeared to be required only very early in the ex vivo cultures, as the leukemic cells isolated from PBMCs that were cultured for 2 days before the separation proliferated spontaneously without monocytes. Once the proliferation was induced, IL-2 signaling seemed to play a central role in maintaining the proliferation, as the addition of an anti-IL-2 antibody inhibited the proliferation of leukemic cell/monocyte cocultures as well as the cultures of ATL cells isolated from 2-day PBMC cultures. This suggested that the efficacy of daclizumab therapy in smoldering/chronic patients was probably due to its blockade of IL-2–dependent proliferation/survival of leukemic cells.

Adherent cells have been shown to be important elements in the spontaneous proliferation of HTLV-1–infected T cells. In patients with HAM/TSP, removal of adherent cells from ex vivo cultures led to a significant reduction in the proliferation manifested by the T cells. Similarly, we have reported previously that IL-9Rα–expressing monocytes contribute to ATL cell spontaneous proliferation in select patients with ATL. However, the requirement for monocytes for ATL cell ex vivo proliferation seems to be a more general phenomenon than implied by their IL-9Rα expression. Here, we demonstrated that purified leukemic cells need autologous monocytes to proliferate (Figure 5A). Furthermore, the interaction between purified leukemic cells and monocytes was contact dependent, as the leukemic cells did not proliferate when put into a different chamber of a Transwell from the monocytes (Figure 6A). Moreover, contrary to the observation of others, exogenously added cytokines, such as IL-1, IL-6, and IL-12, did not replace the requirement for monocytes, suggesting that monocytes provided signals other than cytokines to the leukemic cells (Figure 6B). Interestingly, a monoclonal antibody to MHC class II inhibited the proliferation induced by leukemic cell monocyte cocultures as well as ATL PBMC cultures (Figure 6C-D). This suggested that MHC class II was probably a participant in the interaction between leukemic cells and monocytes. CD14+ monocytes can be infected by HTLV-1 virus, moreover, in HAM/TSP, CD14+ monocytes provided a signal for virus-specific CD8 cell expansion and cytotoxic T-lymphocyte degranulation in a MHC class I–restricted manner in the ex vivo cultures. Although the virus-specific CD4 T-cell response in HAM/TSP has not been analyzed, it would be interesting to investigate virus-specific CD4 cell expansion in patients with ATL.

IL-9 is a Th2 cytokine. IL-9 was produced in more than 80% of smoldering/chronic ATL ex vivo PBMC cultures. We have previously shown that HTLV-1 Tax transactivates IL-9 expression in HTLV-1–infected T-cell lines. However, it appears that IL-2 signaling was also needed for the optimal expression of IL-9 in the ex vivo culture of PBMCs from patients with smoldering/chronic ATL. The addition of an antibody to IL-2Rα profoundly inhibited the production of IL-9, while having little effect on IL-2 and Tax expression. Consistent with our observation, Fung et al and Kajiyama et al showed that IL-2 induced IL-9 production in HTLV-1–infected cell lines and allergen-specific human helper T-cell clones. Further analysis of the IL-9 promoter would be helpful to identify transcriptional elements and epigenetic events that are responsible for IL-2–induced IL-9 expression.

Taken together, our study demonstrated that PBMCs from patients with smoldering/chronic ATL proliferated spontaneously ex vivo in a cytokine-dependent manner. Both IL-2 and IL-9, through the interaction of IL-2 with IL-2Rα, are expressed by the ATL cells and are involved in the monocyte-dependent proliferation of ATL leukemic cells. IL-2Rα–directed therapy (anti-Tac, daclizumab) of patients with smoldering and chronic ATL has been conducted with partial or complete remission in a few patients. However, in light of the fact that both IL-2 and IL-9 are involved in the proliferation of ATL cells, it is difficult to completely inhibit the proliferation with a single cytokine-receptor–directed monoclonal antibody. IL-2, IL-9, and IL-15 share the use of the common γ-chain and the Jak3/STAT5-signaling pathway. Based on these findings, it would be interesting to test agents that target Jak3 in the treatment of patients with smoldering/chronic ATL.

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Authorship

Contribution: J.C. designed and performed research, collected, analyzed and interpreted data, and wrote the manuscript; M.P., R.B.R., V.P.N., and C.K.G. provided patient care and collected patient samples; R.B. provided critical insights in research design; and T.A.W. designed research and revised the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Thomas A. Waldmann, Bldg 10, Rm 4N115, 10 Center Dr, National Institutes of Health, Bethesda, MD 20892-1374; e-mail: twald@helix.nih.gov.

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