Cutaneous T-cell lymphoma (CTCL) refers to a group of diseases characterized by infiltration of malignant and activated T cells in the skin (1). Mycosis fungoides is the most common form with a relatively indolent course and low mortality rate. Patients with mycosis fungoides develop predominantly erythematous patches and plaques, although erythroderma, lymph node involvement, and organ involvement can occur in advanced stages. Sezary syndrome is another frequent variant, presenting with erythroderma and a large number of circulating malignant lymphocytes. Mortality in CTCL is most commonly a result of disease-related immunosuppression.

Skin lesions have an infiltration of atypical lymphocytes. In early disease, many of these atypical lymphocytes seem to be activated reactive lymphocytes, but in more advanced disease a more widespread immune system dysregulation than was previously suspected.

Over the past 1 or 2 years, we have noticed that peripheral blood samples of CTCL patients frequently have neutrophils that fail to separate completely from the peripheral blood mononuclear cells (PBMC) using density gradient separation. This can result from an increased number of activated neutrophils that have a reduced buoyant density ("hypodense" neutrophils; ref. 3). We did not see this with normal healthy controls drawn and processed under identical conditions. Based on this finding of hypodense neutrophils in numerous CTCL blood samples, we formally tested the hypothesis that peripheral blood neutrophils are activated in CTCL and here show that this hypothesis seems to be true. In addition to a density shift, we found that peripheral blood neutrophils in CTCL patients are primed for generation of an enhanced respiratory burst and have an activated surface marker phenotype.
Abnormalities in peripheral blood neutrophils of CTCL patients have not been previously reported. However, previous work has shown CXCL8/interleukin-8 (IL-8) production in CTCL skin lesions (4, 5) and by clonal CTCL cells (6, 7), so we further tested for elevated plasma IL-8 levels. The activated neutrophils could, in turn, generate elevated levels of leukotriene B4 (LTB4), which has recently been shown to enhance recruitment of T cells to inflamed tissues, including skin (8, 9); thus, we tested plasma LTB4 levels as well. Plasma levels of both IL-8 and LTB4 showed significant elevations in CTCL subjects compared with normal controls, providing an initial suggestion as to how activated neutrophils may contribute to the pathophysiology of CTCL.

Materials and Methods

Subjects. Forty-four subjects with a biopsy-confirmed diagnosis of CTCL were recruited from the Cutaneous Oncology Clinic of the Dana-Farber Cancer Institute and Brigham and Women’s Hospital, and informed consent was obtained according to an institutional review board–approved study protocol. Fifteen healthy control volunteers were also recruited for comparison. For initial studies, frozen aliquots of a previously acquired set of blood samples from 34 CTCL subjects and 14 normal controls were used (these were also acquired according to an institutional review board–approved study protocol). Occasional subjects or controls were prospectively excluded because of viral upper respiratory tract infection, suspected or proven skin lesion superinfection, or any other active infection that was felt could potentially activate neutrophils. Skin-directed treatments for stage I and II disease included topical corticosteroids, topical nitrogen mustard, narrow-band UVB, psoralen plus UVA, and topical bexarotene. Most subjects receiving systemic therapy were treated with extracorporeal photopheresis and/or oral bexarotene, although IFN-α-2b, corticosteroids, and denileukin difitox were also used in some subjects.

Blood processing. For initial studies, heparinized venous whole blood was centrifuged (250 × g, 20 minutes, room temperature) through Ficoll-Hypaque (1.077 g/mL, Histopaque; Sigma, St. Louis, MO) and the interface cells were collected and washed once with room temperature Dulbecco’s PBS without divalent cations (Invitrogen, Carlsbad, CA), followed by storage over liquid nitrogen in medium with 10% human serum AB and 10% DMSO. For all subsequent studies, venous blood was drawn from the antecubital vein through a 21-G needle into heparinized Vacutainer tubes (BD, Franklin Lakes, NJ). There was a delay between blood drawing and processing of CTCL samples (average 2.5 hours), and we matched this delay (and also the needles and tubes) when processing normal control samples (average delay 2.8 hours). Blood was maintained at room temperature until processing because temperature shifts are known to cause neutrophil activation. Blood was diluted 1:1 with room temperature Dulbecco’s PBS and spun through a two-layer discontinuous Percoll gradient of 1.080 and 1.100 g/mL densities. The 1.080 g/mL interface yields PBMCs and hypodense neutrophils, and the 1.100 g/mL interface yields normodense granulocytes. Interface cells were recovered and diluted directly in buffers for flow cytometry and chemiluminescence assays. We avoided further washing of the cells because pelleting neutrophils can result in activation.

Plasma for the LTB4 and IL-8 assays was recovered as the upper layer of the Percoll centrifugation and centrifuged at 10,000 × g for 10 minutes at 4°C, then stored at –80°C before use in ELISA assays.

Flow cytometry. Two-color flow cytometric analysis was done on three fractions per subject [whole blood, PBMC with hypodense polymorphonuclear (PMN) and normodense PMN fractions] by using the following monoclonal antibodies against neutrophil surface markers: CD16-phycocerythrin–conjugated antibody, CD11b-FITC–conjugated antibody, CD66b-FITC–conjugated antibody (BD Biosciences, San Diego, CA), CD66b-FITC–conjugated antibody (Beckman...
Coulter, Fullerton, CA), and appropriate isotype-matched control antibodies. Measurements were made using a FACScan flow cytometer (BD Biosciences) with CellQuest software. Whole blood staining was done using PharmLyse buffer (BD PharMingen, San Diego, CA) according to the protocol of the manufacturer.

Chemiluminescence assay. Following separation over Percoll, cells in each fraction were stained with gentian violet in 1% acetic acid and counted using a hemacytometer. The chemiluminescence assay was done using 20,000 neutrophils diluted in 1 mL of Dulbecco’s PBS (with 9 mmol/L calcium, 4.9 mmol/L magnesium, and 0.1% glucose) with 150 μmol/L luminol and 10⁻⁷ mol/L fMLP (Sigma). Peak chemiluminescence was measured for each fraction (whole blood, hypodense fraction, and normodense PMN fraction) using a Wallac Trilux 1450 MicroBeta Liquid Scintillation and Luminescence Counter (Wallac Oy, Turku, Finland) using MicroBeta software.

ELISA assays. LTB₄ levels were determined using the average value of two dilutions (1:10 and 1:5) of purified plasma from CTCL subjects and normal controls using a LTB 4 EIA kit (Cayman Chemical, Ann Arbor, MI) according to the instructions of the manufacturer. IL-8 levels were determined for CTCL subjects and normal controls according to the instructions of the manufacturer using two ELISA kits, which had different measurable ranges [IL-8 Ultrasensitive kit (Biosource International, Camarillo, CA) and human IL-8 ELISA kit (R&D Systems, Minneapolis, MN)].

Statistics. Significance for differences between data sets was tested by the Mann-Whitney nonparametric test using a cutoff value of P < 0.05. Correlations were analyzed using the Spearman correlation coefficient with a cutoff value of P < 0.05. Calculations were done using GraphPad Prism software (GraphPad Software, San Diego, CA).

Results

Cutaneous T-cell lymphoma subjects have increased numbers of hypodense neutrophils. Figure 1A shows representative fluorescence-activated cell sorting plots of our initial observation of an increased number of hypodense neutrophils localizing with the interface cells (PBMC) of a CTCL patient (bottom) compared with a normal control (top). CD16 staining was done to confirm the presence of neutrophils (Fig. 1B), as was Wright-Giemsa staining of cytospin preparations (data not shown). Flow cytometry in some CTCL subjects also showed CD16-negative hypodense granulocytes that were confirmed as eosinophils by Wright-Giemsa staining (data not shown).

We next measured the number of hypodense neutrophils as the percentage of neutrophils (defined as high scatter, CD16⁺ cells) in the interface cell preparation using frozen samples prepared with Ficoll-Hypaque (Fig. 2A). This shows a significantly larger number of hypodense neutrophils in CTCL subjects compared with normal controls. For comparison, a similar fraction of hypodense neutrophils was seen in patients with advanced metastatic adenocarcinoma (3).
Because Ficoll-Hypaque is reported to be slightly activating for neutrophils (10), and also to isolate both hypodense and normodense fractions of neutrophils, subsequent studies used Percoll gradients for separation of fresh blood samples. We were also careful to minimize the time between blood drawing and cell separation and match this time for the control blood.

Figure 2B shows that results with fresh blood specimens separated over Percoll were very similar to results with frozen samples separated over Ficoll-Hypaque (Fig. 2A).

This mechanism of this type of density shift has not been reported in neutrophils; however, in eosinophils, this is believed to result from a combination of cell swelling and degranulation (11–13). To investigate these effects, we measured the scatter properties of neutrophils in whole blood following CD16 staining. We used backgating of the CD16+ neutrophil population to measure mean forward scatter and side scatter of neutrophils and found that mean forward scatter was significantly higher for CTCL subjects than normal controls (675.7 ± 9.8 versus 614.6 ± 11.6, P = 0.001, data not shown). Mean side scatter, on the other hand, was not different. These results suggest that cell swelling is a more prominent contributor to the density shift than degranulation.

Figure 4A shows the results of the chemiluminescence assay and Fig. 3B shows flow cytometry analysis of surface marker expression in whole blood. This analysis also showed a single bell-shaped distribution of forward scatter values in CTCL neutrophils rather than two peaks. This indicates that there is no subset of hypodense neutrophils that is distinct from a majority normodense population and that the cutoff density of 1.080 g/mL selects the least dense fraction of neutrophils from a continuous distribution that is shifted with activation. Similarly, cell surface marker expression analysis in whole blood showed shifted distributions of staining intensity in CTCL rather than the development of a separate subpopulation of activated neutrophils (data not shown). Because density and activation varies on a continuous distribution, the number of neutrophils defined as hypodense is arbitrary. We chose to use a commonly used Percoll density of 1.080 g/mL for separation, which showed ~10% of CTCL neutrophils have a lower density than 1.080 g/mL. A higher Percoll density (e.g., 1.085 g/mL) will yield a greater percentage of neutrophils copurifying with PBMCs.

**Hypodense neutrophils are primed and activated in cutaneous T-cell lymphoma subjects.** The properties of hypodense neutrophils have not been widely reported in the literature, but by analogy to hypodense eosinophils, likely reflect increased neutrophil activation. The functional activity of neutrophils is modulated on several levels. Two particularly important aspects are the “priming” of neutrophils to undergo a respiratory burst and the activation of neutrophils to mobilize granule contents. Priming is shown in assays of the production of reactive oxygen species, such as chemiluminescence or cytochrome reduction. In these assays, unprimed neutrophils generate little activity on low-level stimulation, whereas primed neutrophils will generate a larger burst. Degranulation can be measured by observing released proteases or by the mobilization of granule membrane proteins to the cell surface. Examples of the latter are CD66b, which is detected on the cell surface primarily after granule mobilization, and CD11b (a component of the integrin Mac-1), which is expressed on resting neutrophils and also mobilized in larger amounts to the surface from granules upon activation. CD62L (the adhesion molecule L-selectin) is proteolytically shed from the surface of neutrophils on activation; thus, CD62L expression decreases with activation.

Because hypodense neutrophils are primed and/or activated using separated fractions of hypodense and normodense neutrophils from CTCL subjects. Figure 3A shows results of the fMLP-stimulated chemiluminescence assay and Fig. 3B shows flow cytometry analysis of surface marker expression, confirming that the hypodense fraction is more highly primed as well as more highly activated than the normodense fraction.

**Neutrophils in whole blood of cutaneous T-cell lymphoma subjects are primed and activated compared with normal controls.** Figure 4A shows the results of the chemiluminescence assay using whole blood of CTCL subjects and normal controls. This shows a significant difference in priming for respiratory burst production between CTCL subjects and normal controls. Figure 4B shows results of examining the same samples for surface marker expression by flow cytometry. All three surface markers, CD11b, CD66b, and CD62L, show significant differences, indicating activation of CTCL patient neutrophils compared with normal controls.

**Subgroup analysis of cutaneous T-cell lymphoma subjects.** Figure 5 shows neutrophil activation (as measured by the fraction of hypodense neutrophils) broken down by stage of...
CTCL as well as by treatment category. In our clinic, patients with advanced-stage disease (i.e., stages III or IV) are generally treated with systemic therapy, such as extracorporeal photopheresis, oral bexarotene, oral corticosteroids, or s.c. IFN, whereas those with early-stage disease (i.e., stages I or II) are treated with skin-directed treatments, such as topical corticosteroids or light therapy (psoralen plus UVA or narrow-band UVB). Because of this, the apparent decrease in peripheral blood PMN activation in advanced stages may reflect a confounding suppressive effect of systemic therapy. We have also considered the possibility that light therapy could have a neutrophil-activating effect, but there is no significant difference in neutrophil activation comparing light therapy and other forms of skin-directed therapy in early-stage patients (data not shown).

Plasma leukotriene B₄ and interleukin-8 levels. We next addressed the question of the production of cytokines related to the neutrophil activation in CTCL. Figures 6A and B show plasma LTB₄ and IL-8 levels, demonstrating significant differences between CTCL subjects and normal controls, as well as between skin-directed treatment (primarily stage I/II) and systemic treatment (primarily stage III/IV). We also analyzed the correlation between LTB₄ levels and neutrophil activation in CTCL subjects (as measured by the percentage of hypodense neutrophils shown in Fig. 2B). A significant correlation was found (Spearman r = 0.62, P < 0.0001, data not shown). Similarly, plasma IL-8 levels were significantly correlated with the percentage of hypodense neutrophils (Spearman r = 0.36, P = 0.02). Although statistically significant, these correlations were not as strong as we expected. This may indicate that there is a disconnect in the timing of cytokine production relative to neutrophil activation or that there may be additional cytokines closely linked with neutrophil activation.

Discussion

In summary, these results show that CTCL subjects have significant activation of peripheral blood PMNs as measured by a shift in buoyant density, respiratory burst priming, and surface marker expression. This neutrophil activation is present even in early-stage disease. Plasma IL-8 and LTB₄ levels are also elevated in CTCL subjects, suggesting that neutrophil and T cell feedback via these mediators may contribute to CTCL pathophysiology.

Neutrophil activation in CTCL is potentially important for several reasons. First, these data suggest that systemic priming and activation of neutrophils are present even in early-stage disease, emphasizing that the often mild clinical skin disease occurs in the context of significant systemic immune system dysregulation. Although we were initially surprised by this finding, it is consistent with our previous demonstration of significant contractions in the T-cell receptor repertoire in early-stage CTCL (2). Another group has also observed a suggestion of innate immunity differences between CTCL patients and normal healthy controls (14). In their experiments using a CpG Toll-like receptor 9 agonist and other stimuli to generate a therapeutic antitumor response in CTCL, CTCL patients had a blunted response to the Toll-like receptor 9 agonist, IFN-α, and IL-15 when compared with normal healthy controls.

Second, the presence of activated neutrophils or elevations in IL-8 or LTB₄ could potentially correlate with prognosis or other clinical variables in the same way that eosinophilia and infiltration of skin lesions with eosinophils are markers of a poor prognosis (15, 16). The mechanism that underlies eosinophil activation is unknown but presumably reflects a cytokine imbalance associated with Th2 skewing, and such a cytokine imbalance could be related to priming and activation of neutrophils as well. Correlating these or other related measures with clinical variables will be a focus of future work. At this point, it has proved difficult to make such correlations because we included a highly heterogeneous patient population with a variety of clinical presentations and treatments as well as varying clinical courses (i.e., disease that was improving, worsening, or staying the same) at the time of blood sampling. As shown in Fig. 5, there seems to be a consistent trend toward less neutrophil activation in advanced...
stages, but advanced-stage disease is closely linked with the use of systemic treatments. Because the subjects with advanced CTCL that we studied were all actively receiving systemic treatments, it is not possible to determine whether the lower levels of neutrophil activation seen in our CTCL subjects with advanced disease are attributable to disease stage or to disease treatment. We believe it is likely that this decrease in neutrophil activation is due to systemic treatments with immunosuppressive effects, but will need to confirm this in future work by testing advanced-stage subjects before treatment. As shown in Figs. 5 and 6, there also seems to be less neutrophil activation, IL-8 and LTB4, in subjects who are in remission compared with those having clinically evident active disease at the time of the blood draw. Nonetheless, subjects in clinical remission still showed somewhat higher levels of activated neutrophils compared with normal controls.

Third, the activated neutrophils could be directly involved in the pathophysiology of CTCL. There are many possibilities for such involvement (17). The elevated LTB4 levels shown in Fig. 6A are one reasonable possibility and this is supported by the recent finding that LTB4 can modulate the adhesive properties of CD4+ T cells expressing the high affinity LTB4 receptor (BLT1; ref. 8). In a mouse model of skin inflammation, LTB4 was a key mediator of T cell homing to skin (9), and LTB4 is therefore an attractive potential contribution to the skin tropism exhibited in CTCL. It is interesting that this would likely be a systemic effect rather than local production of LTB4 because there are generally relatively few neutrophils in CTCL skin lesions. The elevations in plasma IL-8 shown in Fig. 6B may indicate a mechanism for systemic neutrophil priming and activation in CTCL. However, it is important to note that detailed in vitro culture experiments will be required to verify that the observed concentrations of IL-8 (and likewise LTB4) are sufficient to produce the observed effects. Also of potential importance is the oxidative stress associated with neutrophil activation. Reactive oxygen species can lead to T-cell dysfunction (3) and could be related to the immunosuppression and loss of T-cell receptor diversity seen in CTCL.

Activation of peripheral blood neutrophils is seen in several disease processes and is not specific to CTCL. Among skin diseases, psoriasis and dermatitis herpetiformis show evidence of systemic neutrophil activation (18–20), but not atopic dermatitis or superficial skin cancer (19). This correlates well with the neutrophilic infiltrates in psoriasis and dermatitis herpetiformis skin lesions but not in atopic dermatitis and skin cancers. Advanced adenocarcinoma patients also show systemic activation of neutrophils, which correlates with impaired T-cell function in these patients (3). Although these various disease processes are associated with the presence of neutrophil activation, it is likely that the mechanisms involved in each case are different, especially with regard to neutrophil-T cell interactions. Further work investigating the causes and effects of neutrophil activation in CTCL should lead to significant added insight into this complex disease.

Acknowledgments

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