The role of eosinophils in the pathobiology of Hodgkin's disease

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Summary

Background: Even though the presence of a prominent tissue eosinophilia represents a common histopathologic feature of Hodgkin's disease (HD), eosinophils have been mainly regarded as 'innocent' bystanders recruited and activated during the cellular reaction typical of HD. To evaluate the putative role of eosinophils or eosinophil-derived cytokines on tumor-cell regulation in HD, we have analyzed these cells for the functional expression of surface ligands (L) of the tumor necrosis factor (TNF) superfamily, whose specific receptors are known to transduce proliferation signals at the surface of Hodgkin (H) and Reed-Sternberg (RS) cells.

Materials and methods: Eosinophils from peripheral blood of healthy donors and patients with HD, primary hyper eosinophilic syndrome (HES), or secondary hyper eosinophilia (HE), were purified by density gradient centrifugation and immunomagnetic depletion of residual granulocytes.

Results: By immunostaining and mRNA analysis, we were able to show that eosinophils from normal donors and patients with HD, HES, and HE express a number of receptors and ligands of the TNF superfamily, including CD40, CD40L, CD30L, CD95/Fas, CD95/FasL and 4-1BB. In addition, we provide evidence that cytokines regulating eosinophil proliferation and activation, i.e., interleukin (IL)-5, IL-3, and granulocyte-macrophage colony-stimulating factor, are able to enhance the cellular density of several TNF superfamily ligands and/or receptors at the surface of cultured eosinophils. Finally, we have shown that native CD40L and CD30L at the surface of purified eosinophils are functionally active and able to transduce proliferative signals on CD40+ and CD30+ target cells, including cultured H–RS cells.

Conclusions: Our data suggest that eosinophils may act as important elements in the pathology of HD by providing cellular ligands for TNF-superfamily receptors (CD40, CD30, CD95/Fas) able to transduce proliferation and antiapoptotic signals at the surface of H–RS cells. The presence of eosinophils as receptors for TNF ligands expressed by activated T cells (i.e., OX40L, Fasl, CD40L, 4-1BB), also suggest that eosinophils may contribute to the deregulated network of interactive signals between H–RS cells, T cells, and other surrounding reactive cells.

Key words: eosinophils, Hodgkin's disease, Reed–Sternberg cells, TNF-like ligands

Introduction

Hodgkin's disease (HD) is characterized by the presence of the malignant mononucleated Hodgkin (H) and multinucleated Reed–Sternberg cells (usually accounting for 1% to 2% of the tumor mass) embedded in an abundance of hyper reactive cells, including T and B lymphocytes, histiocytes, plasma cells, stromal cells, neutrophils, and eosinophils [1, 2]. Several lines of evidence indicate that proliferation of H–RS cells is regulated by a complex network of cytokine-mediated and cell contact-dependent interactions among tumor cells and the reactive cell populations accumulating in HD-involved tissues [1–5]. In this regard, CD4+ T lymphocytes and other reactive cells, following recruitment and functional activation by tumor cell-derived cytokines, have been shown to interact with H–RS cells and provide in turn soluble and membrane-associated growth factors regulating tumor cell growth and expansion [3–8].

Among the surface molecules mediating cell contact-mediated interactions of H–RS cells with surrounding reactive cells, receptors (R) and ligands (L) of the tumor necrosis factor (TNF) superfamily [9, 10] have recently been shown to act as important elements in the pathobiology of HD [5, 8]. The TNF-receptor superfamily consists at present of 10 transmembrane type I glycoproteins, including the p75 NGFR, the p60 TNFR-type I (CD102a), the p80 TNFRII (CD102b), TNFR-type III (TNFR-RII), CD27, CD30, CD40, CD95/Fas, OX40, and 4-1BB [9, 10]. The interaction of these receptors with their specific ligands (i.e., TNF, LT-α, LT-β, CD27L, CD30L, CD40L, CD95L/Fas-L, OX40L, 4-1BBL, and the TNF-related apoptosis-inducing ligand) results in the transduction of signals leading to either cell activation and proliferation or cell death, depending on the cell type and the different pathways of intracellular signaling [5, 9, 10].

The presence of a prominent eosinophil infiltration in tissues involved by HD was described as early as one century ago [11–13], and subsequent studies have shown that intact eosinophils or eosinophil-degranulation products (i.e., eosinophil peroxidase or eosinophil basic protein) can be detected in up to 89% of nodular sclerosis
(NS) and mixed cellularity (MC) HD cases [13–17]. Conversely, tissue eosinophilia represents a very infrequent finding in the lymphocyte predominant (LP) subtype of HD [13, 14]. The massive recruitment of eosinophils within HD-involved tissues has been explained by the finding that H–RS cells are able to synthesize interleukin (IL)-5 [3, 4, 13, 18, 19] and granulocyte-monoocyte colony-stimulating factor (GM-CSF) [3, 4, 20–22], which are potent stimulators of eosinophil growth and functional activation [23–26]. The extensive presence of IgE deposits within H–RS cells and on the surrounding cells and connective tissues, as detected in most HD cases, may also account in part for the typical eosinophilia of HD [27].

In addition, the presence of IL-5 mRNA was also demonstrated by *in situ* hybridization in reactive T lymphocytes surrounding H–RS cells in HD cases with eosinophilia [3, 13, 18, 19], suggesting that eosinophil recruitment may be a final result of the complex cytokine cascade typical for HD.

Despite the extensive presence of eosinophils in HD tissues, these cells have been mainly regarded as 'innocent' bystanders, and their putative role in the regulation of H–RS cell growth and functional activation has not been addressed until now. To clarify the pathobiologic significance of eosinophils in HD, we have analyzed these cells for the functional expression of TNF superfamily ligands, whose specific receptors are known to transduce proliferation and/or antiapoptotic signals at the surface of H–RS cells. In addition, we have evaluated the presence on eosinophils of TNF-superfamily receptors able to engage ligands presented by hyperreactive T cells within HD tissues.

**Materials and methods**

**Eosinophil isolation and purification**

Eosinophils were obtained from peripheral blooduffy coats of consenting healthy donors (*n* = 5), patients with HD (*n* = 4), primary hypereosinophilic syndrome (HES) (*n* = 3), and secondary hypereosinophilia (HE) (*n* = 2). Eosinophil isolation was performed by combining density gradient centrifugation with the removal of contaminating neutrophils by negative immunomagnetic selection with anti-CD16 monoclonal antibodies (MoAbs), as described [28, 29]. Briefly, 2.25% dextran sedimentation cells were then washed and centrifuged through six isotonic (osmolality of 285 to 294 mOsm/kg) discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradients, with densities ranging from 1.077 g/ml to 1.105 g/ml [29]. Interfaces at a specific gravity of 1.090, 1.095, and 1.105 g/ml were collected and depleted of contaminating neutrophils by incubation with the anti-CD16 MoAb CLB/FcR gran 1 (IV International Workshop, Inlaco, Milan, Italy) followed by sheep antismouse IgG-coated immunomagnetic beads (Dynabeads, Dynal, Norway) [29]. Ninety-five percent to 98% pure eosinophil preparations were either fixed in 0.5% paraformaldehyde or resuspended in Iascone's modified Dulbecco medium (IMDM, Gibco, Paisley, Scotland) supplemented with 5% fetal calf serum (FCS, Gibco).

**Monoclonal antibodies and flow cytometry**

The monoclonal antibodies utilized in the study, i.e., anti-CD40 M44, anti-CD40L M90, anti-CD30 M44, anti-CD30L M80, anti-CD95/Fas M3, anti-4-1BB M13 (kindly provided by Immunex Research and Development Co., Seattle, WA, USA) and anti-CD60 A255 (Ansecell, Byport, MN, USA) were identified and characterized as described [30–34]. Indirect immunofluorescence was performed by sequentially incubating cells with saturating concentrations (10 μg/ml) of each MoAb and phycoerythrin (PE)-conjugated F(ab')2 fragments of goat anti-mouse Ig (H + L) (Jackson Immuno Research Lab., West Groove, PA, USA), as described [35, 36]. Nonspecific binding of monoclonal antibodies was assessed by labeling cells with isotype-matched control mouse IgGs (Jackson Immuno Research Laboratories). Viable, antibody-labeled cells were identified according to their forward and right angle scattering, electronically gated and analyzed for surface fluorescence on a FACSScan flow cytometer (Becton Dickinson). Phenotype and characteristics of the human cell lines DG-75 (B-NHL), KE37 (T-NHL), RI-1 (B-NHL), HL-60 (AML), ML3 (AML) FLG291 (osteoclasts), DLD-1 (colonic carcinoma), of the HD-derived cell line HDLM-2 and of the anaplastic large cell lymphoma (ALCL) cell line Karpas 299, have been described in detail previously [22, 35–37]. These cells were utilized as positive (+) or negative (−) controls for immunostaining procedures, RNA analysis and functional studies. A subclone (J-L) of the Jurkat T-cell line, isolated in our laboratory and showing a constitutive expression of CD40L [35], was utilized as a positive control for CD40L.

**RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)**

Total RNA (1 μg), extracted by the guanidium thiocyanate method [38], was reverse-transcribed by avian myeloblastosis virus (AMV) reverse transcriptase (Promega Co., Madison, WI, USA) in a 20 μl reaction mix containing hexodeoxyribonucleotides random primers (0.4 μg) for 1.0 hours at 42 °C [26]. Two microliters of the same cDNA preparations were amplified in a 50 μl volume of final reaction mix by a Perkin Elmer 9600 thermal cycler, with 25 pmol of primers specific for CD40, CD40L, CD30, CD30L, CD95/Fas, CD95L/FasL. Primer sequences, corresponding regions, size of the expected amplified products, and PCR conditions are listed in Table 1. In addition, B-actin primers (Clontech Laboratories Inc., Palo Alto, CA, USA) were utilized as internal controls for RT-PCR reactions, according to the manufacturer's instructions. In some experiments, 10 μl aliquots of amplified products specific for CD40 and CD40L were removed after 25, 30, or 35 cycles and analyzed separately. In all instances, 10 μl of amplified cDNA products were run in 1.5% agarose gels and analyzed under UV light following ethidium bromide staining.

**In vitro cultures and proliferation assay**

Purified eosinophils (2.0 × 10^5/ml) were cultured for 24 and 72 hours in IMDM supplemented with 5% FCS in the presence of a combination of granulocyte-macrophage colony-stimulating factor (GM-CSF, 100 ng/ml), interleukin (IL)-3 (500 U/ml), and IL-5 (10 ng/ml). All recombiant cytokines were obtained from Genzyme Co. (Cambridge, MA, USA). The CD40+ RI-1 B-cell line and CD30+ cell lines KE37, HDLM-2, and Karpas 299 were utilized as responsive target cells to investigate the capability of native CD40L and CD30L on human eosinophils to transduce CD40-mediated and CD30-mediated growth signals [30–32, 39]. Purified eosinophils (100 × 10^6/well) were fixed in 0.5% paraformaldehyde as described [29, 30, 33], and cocultured for 72 hours with R1-1, KE37, HDLM-2, or Karpas 299 cells (1 × 10^5) in 96-well U-bottomed microplates. Control experiments were performed in the presence of an excess (10 μg/well) of CD40-Fc or CD30-Fc fusion proteins to specifically block CD40-CD40L and CD30-CD30L interactions [30, 41]. In some experiments, CD40/CD40L interactions were blocked by the anti-CD40 MoAb 89, as described [35]. The CD30− and CD40− cell line ML-3 was employed as a further negative control. Quadruplicate cultures were pulsed with 1 μCi/well ^3H-thymidine (H-TdR, specific activity 25 Ci/mmol; Amersham International, Amersham Place, UK) for the final 12 hours of culture, harvested on glass fiber membranes, and counted in a liquid scintillation beta counter (Tri-Carb 1600TR, Canberra-Packard).
Results

Expression of TNF-superfamily ligands and receptors on purified human eosinophils

The constitutive expression of TNF-superfamily ligands and receptors on purified human eosinophils was assessed by flow cytometry and RNA studies. In addition, to evaluate the effects of cytokines involved in the functional activation of eosinophils for the regulation of the same molecules, eosinophil preparations from normal donors were cultured in the presence of a combination of GM-CSF (100 ng/ml), IL-3 (500 U/ml), and IL-5 (10 ng/ml).

As shown in Figure 1, circulating eosinophils displayed a clear constitutive expression of CD40, CD40L, CD30L, CD95/Fas, and 4-1BB, while lacking surface OX40 and CD30. A 24-hour exposure to the GM-CSF/IL-3/IL-5 combination induced a significant up-regulation of surface CD40, CD30L, and CD95/Fas on cultured eosinophils (Figure 1). The same cytokines also caused slight upregulation of surface CD40L, CD30L and CD95/Fas on cultured eosinophils (Figure 1). The same cytokines also caused slight upregulation of surface CD40L, CD30L and CD95/Fas on cultured eosinophils (Figure 1). Conversely, fresh eosinophils lacked CD30 mRNA and displayed a low level expression of CD95L/FasL transcripts, which appeared down-regulated following culture in the presence of cytokines (Figure 2A). To further assess the effects of eosinophil-activating cytokines on the regulation of CD40 and CD40L at RNA level, a semi-quantitative RT-PCR approach was employed. To this end, RNAs obtained from eosinophils cultured for different times in the presence of GM-CSF plus IL-3 for one and three days, resulted in maintenance or the slight increase of the constitutive mRNA levels for CD30L and CD95/Fas (Figure 2A). Conversely, fresh eosinophils lacked CD30 mRNA and displayed a low level expression of CD95L/FasL transcripts, which appeared down-regulated following culture in the presence of cytokines (Figure 2A). To further assess the effects of eosinophil-activating cytokines on the regulation of CD40 and CD40L at RNA level, a semi-quantitative RT-PCR approach was employed. To this end, RNAs obtained from eosinophils cultured for different times in the presence of GM-CSF plus IL-3, were retrotranscribed and amplified with the appropriate primers (Table I). Aliquots of amplified products were then removed after 25, 30, and 35 RT-PCR rounds, and separately analyzed on agarose gels. As shown in Figure 2B, while CD40 message was detectable on unstimulated eosinophils only following 35 amplification cycles, CD40-specific amplified products were readily detected at 25 and 30 RT-PCR rounds from eosinophils cultured for 24 and 72 hours in the presence of GM-CSF plus IL-3. Similarly, a CD40L-specific message was evident starting from 25 amplification rounds in eosinophils exposed

- PCR conditions for CD30 were 4 min at 94 °C, followed by 35 cycles of 45 sec at 94 °C, 45 sec at 62 °C, 1 min at 72 °C, and a final extension of 7 min at 72 °C.
- PCR conditions for CD30L were 4 min at 94 °C, followed by 35 cycles of 45 sec at 94 °C, 1.5 min at 68 °C, and a final extension of 5 min at 72 °C.
- Primer set for CD40 and CD40L were obtained from Stratagene (La Jolla, CA, USA), and amplification was performed according to manufacturer's guidelines.
- PCR conditions for Fas/CD95 were 4 min at 94 °C, followed by 40 cycles of 45 sec at 94 °C, 1.5 min at 50 °C, 2 min at 72 °C, and a final extension of 7 min at 72 °C.
- PCR conditions for FasL/CD95L were 4 min at 94 °C, followed by 40 cycles of 45 sec at 94 °C, 45 sec at 59 °C, 2 min at 72 °C, and a final extension of 5 min at 72 °C.

Abbreviations: bp = base pairs; S = sense primer; AS = antisense primer.

Table 1. Oligodeoxynucleotides used in RT-PCR reactions.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>cDNA Pos.</th>
<th>Amplified fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD30 S</td>
<td>CTGTGTCCTACCCAATCT</td>
<td>1121-1140</td>
<td>860^a</td>
</tr>
<tr>
<td>CD30 AS</td>
<td>CTTCTTTTCTTCTTCTCCA</td>
<td>1960-1980</td>
<td>689^b</td>
</tr>
<tr>
<td>CD30L S</td>
<td>CCCCTCTGGAGACAGACGC</td>
<td>153-170</td>
<td>43^c</td>
</tr>
<tr>
<td>CD30L AS</td>
<td>CCTGAAGCAGCAAGAAACTG</td>
<td>362-381</td>
<td>292^d</td>
</tr>
<tr>
<td>CD40 S</td>
<td>CAGTCACTGTCTTCTTGT</td>
<td>297-316</td>
<td>1008^e</td>
</tr>
<tr>
<td>CD40 AS</td>
<td>GCCCTGCTCTTCGCAAACCA</td>
<td>996-1015</td>
<td>974^d</td>
</tr>
<tr>
<td>CD40L S</td>
<td>GCCGGAAGGTGCGGGAAGA</td>
<td>297-316</td>
<td>1008^e</td>
</tr>
<tr>
<td>CD40L AS</td>
<td>GACAGTCCCCCTTGAGGTA</td>
<td>1286-1304</td>
<td>91</td>
</tr>
</tbody>
</table>

^a PCR conditions for CD30 were 4 min at 94 °C, followed by 35 cycles of 45 sec at 94 °C, 45 sec at 62 °C, 1 min at 72 °C, and a final extension of 5 min at 72 °C.

^b PCR conditions for CD30L were 4 min at 94 °C, followed by 35 cycles of 45 sec at 94 °C, 1.5 min at 68 °C, and a final extension of 5 min at 72 °C.

^c Primer set for CD40 and CD40L were obtained from Stratagene (La Jolla, CA, USA), and amplification was performed according to manufacturer's guidelines.

^d PCR conditions for Fas/CD95 were 4 min at 94 °C, followed by 40 cycles of 45 sec at 94 °C, 1.5 min at 50 °C, 2 min at 72 °C, and a final extension of 7 min at 72 °C.

^e PCR conditions for FasL/CD95L were 4 min at 94 °C, followed by 40 cycles of 45 sec at 94 °C, 45 sec at 59 °C, 2 min at 72 °C, and a final extension of 5 min at 72 °C.

were more heterogeneous, even though a lower cellular density of CD95 was usually observed on eosinophils from HES patients (Table 2). Conversely, circulating eosinophils from either normal donors or patients never expressed surface CD30 (Table 2).

Expression of mRNA for CD30, CD40, CD95/Fas, and their respective ligands by eosinophils was also studied by RT-PCR. As shown in Figure 2, amplified cDNA products specific for CD30L, CD95/Fas, CD40, and CD40L were detected on freshly isolated eosinophils from normal donors. Culturing of these cells in the presence of GM-CSF plus IL-3 for one and three days, resulted in maintenance or the slight increase of the constitutive mRNA levels for CD30L and CD95/Fas (Figure 2A). Conversely, fresh eosinophils lacked CD30 mRNA and displayed a low level expression of CD95L/FasL transcripts, which appeared down-regulated following culture in the presence of cytokines (Figure 2A). To further assess the effects of eosinophil-activating cytokines on the regulation of CD40 and CD40L at RNA level, a semi-quantitative RT-PCR approach was employed. To this end, RNAs obtained from eosinophils cultured for different times in the presence of GM-CSF plus IL-3, were retrotranscribed and amplified with the appropriate primers (Table 1). Aliquots of amplified products were then removed after 25, 30, and 35 RT-PCR rounds, and separately analyzed on agarose gels. As shown in Figure 2B, while CD40 message was detectable on unstimulated eosinophils only following 35 amplification cycles, CD40-specific amplified products were readily detected at 25 and 30 RT-PCR rounds from eosinophils cultured for 24 and 72 hours in the presence of GM-CSF plus IL-3. Similarly, a CD40L-specific message was evident starting from 25 amplification rounds in eosinophils exposed
Figure 1. Expression of TNF-superfamily ligands and receptors on purified human eosinophils from normal donors, as detected by flow cytometry. More than 95% pure resting (dotted lines) and cytokine-activated (thick lines) eosinophils were stained with 5 μg/ml of MoAbs specific for CD40 (M2), CD40L (M90), CD30 (M44), CD30L (M80), CD95/Fas (M3), 4-1BB (M13), OX-40 (Act35) followed by PE-conjugated goat antimouse antibody. Background fluorescence was determined by staining with isotype-matched control Igs (thin lines). Eosinophils were activated by a 24-hour in vitro exposure to a combination of GM-CSF (100 ng/ml), IL-3 (500 U/ml) and IL-5 (10 ng/ml).

Fluorescence Intensity

Table 2. Expression of ligands and receptors of the TNF superfamily on purified eosinophils from normal subjects and patients with Hodgkin's disease and primary or secondary hypereosinophilia.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>NS (n = 5)</th>
<th>HD (n = 4)</th>
<th>HES (n = 3)</th>
<th>HE (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40</td>
<td>30</td>
<td>35</td>
<td>42</td>
<td>32</td>
</tr>
<tr>
<td>CD40L</td>
<td>22</td>
<td>28</td>
<td>38</td>
<td>25</td>
</tr>
<tr>
<td>CD30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD30L</td>
<td>28</td>
<td>38</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>CD95/Fas</td>
<td>50</td>
<td>61</td>
<td>35</td>
<td>59</td>
</tr>
<tr>
<td>OX40</td>
<td>0</td>
<td>25</td>
<td>38</td>
<td>30</td>
</tr>
<tr>
<td>4-1BB</td>
<td>19</td>
<td>25</td>
<td>38</td>
<td>29</td>
</tr>
</tbody>
</table>

Values are expressed as the difference of mean fluorescence intensity (MFI) between staining of purified eosinophils with the appropriate MoAb and isotype-matched control mouse Igs. Figures represent the mean of net MFI in each group of subjects. SEM never exceeded 15% for each group.

Abbreviations: NS - normal subjects; HD - Hodgkin's disease; HES - primary hypereosinophilic syndrome; HE - secondary hypereosinophilia.

CD40L and CD30L expressed on eosinophils are functionally active

To examine if native CD40L and CD30L expressed at the surface of eosinophils were functionally active, 0.5% paraformaldehyde-fixed purified human eosinophils (100 x 10^6/well) were cocultured with 1 x 10^5 of Ri-1 (CD40+/CD30-), KE37 (CD40-/CD30+), HDLM-2 (CD40+/CD30+), or Karpas 299 (CD40-/CD30+) cells. Previous studies [30, 31, 41] have shown that these cell lines can respond to membrane-expressed recombinant CD30L by being either growth-stimulated (KE37, HDLM-2) or

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growth-inhibited (Karpas 299). In addition, recombinant
cD40L was shown to stimulate proliferation of Ri-1 lymphoma
B cells, but not of the other cell lines. Biological
responses mediated by native CD40L and CD30L ex-
pressed on eosinophils were detected using the ³H-TdR
incorporation assay, and specifically blocked by the addi-
tion of an excess (10 µg/ml) of soluble CD40-Fc or CD30-Fc fusion
proteins or by the blocking anti-CD40
MoAb 89. As shown in Table 3, eosinophils induced a
CD30L-mediated proliferation of the HD-derived cell
line HDLM-2 and of KE37 cells, while inhibiting growth of the
ALCL cell line Karpas 299. These effects were
abolished by blocking CD30L/CD30 interactions by solu-
tions (i.e., IL-6, TNF, LT-α) and increased expression of surface
antigens involved in cell-cell interactions (i.e., ICAM-1/
ML-3 and reactive bystander cells.

Thus, native CD40L and CD30L on human eosino-
phils are a functionally active surface structure able to transduce CD40-
and CD30L-mediated signals, including growth stimulation of cultured H–RS cells.

Table 3. Biological activity of native CD40L and CD30L expressed at
the surface of purified human eosinophils on CD40+ or CD30+ target
cells.

<table>
<thead>
<tr>
<th>Target cells</th>
<th>Phenotype</th>
<th>Eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>CD40-Fc</td>
</tr>
<tr>
<td>Ri-1</td>
<td>CD40+CD30-</td>
<td>+ = +</td>
</tr>
<tr>
<td>KE37</td>
<td>CD40+/CD30-</td>
<td>+ = =</td>
</tr>
<tr>
<td>HDLM-2</td>
<td>CD40+/CD30+</td>
<td>+ = +</td>
</tr>
<tr>
<td>Karpas 299</td>
<td>CD40+/CD30+</td>
<td>= = =</td>
</tr>
<tr>
<td>ML-3</td>
<td>CD40-/CD30-</td>
<td>= = =</td>
</tr>
</tbody>
</table>

100 x 10⁶/well paraformaldehyde-fixed human eosinophils were cocul-
tured with each target cell type (1 x 10⁵) in 96-U-bottomed microplates
for 72 hours. Proliferation of target cells was assessed by pulsing cultures with 0.5 µCi/well of ³H-thymidine for the final 12 hours of
 incubation. Experiments were performed in the absence (none) and in
the presence of 10 µg/ml of soluble CD40-Fc or CD30-Fc fusion
proteins or to specifically block CD40/CD40L and CD30/CD30L inter-
actions.

Discussion
The extensive presence of eosinophils and/or their degra-
dulation products in almost all specimens of NS and MC
HD [13–17, 25] along with the expression of eosinophil-
activating cytokines (i.e., IL-5 and GM-CSF) by H–RS
cells and surrounding T lymphocytes, have been docu-
mented by several studies [3, 18–20, 22]. These findings,
while indicating that eosinophil recruitment and activa-
tion is an important consequence of the deregulated
cellular reaction typical of HD, also support that eosino-
phils accumulating in HD-involved tissues may in turn
have a role in the pathobiology of this lymphoma. None-
theless, while it has been previously shown that reactive T
cells are able to provide cytokines (IL-9, IL-2, IL-6) [2, 3,
5, 19, 42, 43] and cell contact-dependent interactions
(LFA-1/CD11a, CD2, CD28, CTLA-4) [7, 44–46] trigger-
ning proliferation and activation of H–RS cells [2, 3, 6, 8],
no information is currently available as to the putative
role of eosinophils and/or eosinophil-derived cytokines
on tumor cell regulation in HD.

TNF-like ligands are membrane-bound type II glyco-
proteins, usually expressed on activated, but not resting,
T cells and playing an important part in T-B-cell cooperation
and other immunoregulatory functions [5, 9, 10].
These ligands elicit their biologic functions through bind-
ing specific counterreceptors of the parallel TNF-recep-
tor superfamily [9, 10]. Cultured and tissue H–RS cells express the high cellular density of most of the TNF-
superfamily receptors, including CD30, CD40, CD95,
4-1BB, and TNFR type I (CD102a) and type II (CD102b)
[3, 5, 8]. Signals transduced through these receptors have
been shown to be involved in the activation, proliferation,
and survival of cultured H–RS cells. For instance, CD30L
stimulates proliferation of some H–RS cell lines [30, 40,
41], and CD40L, although apparently lacking mitogenic
activity, is able to enhance colony formation of cultured
H–RS in semisolid medium [32, 35]. In addition, we have
previously shown that CD40L ability to enhance colony
growth of H–RS is potentiated by IL-9, a cytokine crit-
ically involved in the biology of HD [42, 47], and that
CD40L is able to rescue from apoptotic degeneration
donor H–RS cells in vitro [35]. CD40L and CD30L-
mediated signaling on H–RS cells also results in a strik-
ing cellular activation with enhanced cytokine secretion
(i.e, IL-6, TNF, LT-α) and increased expression of surface
antigens involved in cell-cell interactions (i.e., ICAM-1/
CD40L, B7-1/CD80, B7-2/CD86) [30, 32, 35, 40]. Since
most of the hyperreactive cell types surrounding tumor
phils accumulating in HD-involved tissues may in turn
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5, 19, 42, 43] and cell contact-dependent interactions
(LFA-1/CD11a, CD2, CD28, CTLA-4) [7, 44–46] trigger-
ing proliferation and activation of H–RS cells [2, 3, 6, 8],
no information is currently available as to the putative
role of eosinophils and/or eosinophil-derived cytokines
on tumor cell regulation in HD.

TNF-like ligands are membrane-bound type II glyco-
proteins, usually expressed on activated, but not resting,
T cells and playing an important part in T-B-cell cooperation
and other immunoregulatory functions [5, 9, 10].
These ligands elicit their biologic functions through bind-
ing specific counterreceptors of the parallel TNF-recep-
tor superfamily [9, 10]. Cultured and tissue H–RS cells express the high cellular density of most of the TNF-
superfamily receptors, including CD30, CD40, CD95,
4-1BB, and TNFR type I (CD102a) and type II (CD102b)
[3, 5, 8]. Signals transduced through these receptors have
been shown to be involved in the activation, proliferation,
and survival of cultured H–RS cells. For instance, CD30L
stimulates proliferation of some H–RS cell lines [30, 40,
41], and CD40L, although apparently lacking mitogenic
activity, is able to enhance colony formation of cultured
H–RS in semisolid medium [32, 35]. In addition, we have
previously shown that CD40L ability to enhance colony
growth of H–RS is potentiated by IL-9, a cytokine crit-
ically involved in the biology of HD [42, 47], and that
CD40L is able to rescue from apoptotic degeneration
donor H–RS cells in vitro [35]. CD40L and CD30L-
mediated signaling on H–RS cells also results in a strik-
ing cellular activation with enhanced cytokine secretion
(i.e, IL-6, TNF, LT-α) and increased expression of surface
antigens involved in cell-cell interactions (i.e., ICAM-1/
CD40L, B7-1/CD80, B7-2/CD86) [30, 32, 35, 40]. Since
most of the hyperreactive cell types surrounding tumor
in HD tissues express a number of TNF-like ligands
and/or receptors (i.e., CD40L, CD30L, CD30, CD40L,
OX40, OX40L, 4-1BB, and 4-1BBL) on CD4+ T cells, CD40,
CD30L, and 4-1BBL on monocyte/macrophages and his-
tiocytes, CD40 and CD30L on B cells), either constitu-
tively and/or following cellular activation [5, 8, 9, 10, 30–
32, 41], it appears that ligands and receptors of the TNF
superfamily may play an important role in the deregu-
lated network of interactive signaling between H–RS cells and
reactive bystander cells.

In the present study, we have shown that resting and
cytokine-activated eosinophils may express in turn a
number of TNF-like ligands and receptors, enabling these
cells to take an active part in the unbalanced cytokine
network and cell contact-dependent activation cascade
typical for HD. As detected by RNA and immunostain-
ing studies, highly purified human circulating eosinophils
express CD40, CD40L, CD30L, CD95/Fas, CD95L/FasL,
4-1BB, and in some instances, OX40. The surface density
of some TNF-superfamily ligands (CD40L, CD30L) or
receptors (CD40, 4-1BB) on eosinophils from HD and
HES patients was significantly higher in comparison to purified eosinophils from normal donors, probably reflecting a cytokine-mediated activation of eosinophils in these pathologic conditions [25, 26]. Previous in vivo studies have demonstrated a selective enhancement of IL-5, IL-3, and/or GM-CSF levels in the sera and tissues of patients with primary or secondary hypereosinophilia [49-52] and HD [25, 53]. Accordingly, we have shown here that a combination of the eosinophils-activating cytokines IL-5, IL-3, and GM-CSF is able to enhance CD40, CD40L, CD30L, and 4-1BB expression on purified eosinophils from normal subjects. Based on these findings, it might be concluded overall that the concerted action of cytokines leading to eosinophil functional activation may concurrently up-regulate some TNF-like ligands (CD40L, CD30L) and/or receptors (CD40, 4-1BB, OX40) at the eosinophil surface both in vitro and in vivo.

To evaluate whether native CD40L and CD30L expressed at the surface of human eosinophils were functionally active in transducing CD40-mediated and CD30-mediated signals, a number of CD40+ and CD30+ target cell lines were incubated in vitro with paraformaldehyde-fixed purified eosinophils. These studies demonstrated that CD30 engagement by eosinophil-expressed CD30L is able to induce proliferation of the HD-derived cell line HDLM-2 and to cause a significant growth inhibition of Karpas 299 ALCL cells. These effects were highly specific, being abolished by an excess of soluble CD30 protein, and completely overlapping the biologic activity of recombinant human CD30L, previously shown to exert a mitogenic or antiproliferative action on HDLM-2 and Karpas 299 cells, respectively [30, 31, 41]. Similarly, we have shown that membrane CD40L on human eosinophils is functionally active in triggering CD40-dependent proliferation of lymphoma B cells.

Further, present and previous findings that eosinophils express some of the TNF-superfamily receptors and may represent another important cellular source of functionally active CD40L, CD30L, and CD95L/FasL [54, 55] for H-RS cells and/or other reactive cells within the HD microenvironment raise the issue of the pathobiologic significance of eosinophilic infiltration in HD tissues. Previous data on TNF-like ligand expression by hyperreactive normal cells accumulating within HD-involved areas have pointed to the participation of most of these ligands in paracrine stimulatory loops in HD [5, 8, 30-32, 35]. As indicated in Figure 3, it therefore appears likely that eosinophils, recruited and activated by H-RS cell- and T-cell-derived cytokines (i.e., IL-5, IL-3, and GM-CSF), may in turn up-regulate surface TNF ligands (i.e., CD40L and CD30L) and interact with H-RS cells to transduce CD40- and CD30-mediated signals leading to proliferation, apoptotic rescue, and cellular activation of tumor cells. Whether a cytokine-related down-regulation of CD55L/FasL on activated eosinophils may lead to an additional growth advantage for CD95/Fas-expressing H-RS cells remains to be established. In addition to malignant cells, activated T lymphocytes may represent another putative cellular target for eosinophil interactions within HD tissues. Activated CD4+/CD45R0+ T cells, which represent the main reactive cellular component surrounding and functionally interacting with H-RS cells, in fact display the appropriate surface counterstructures (i.e., CD40L, CD30L, 4-1BBL, OX40, OX40L, CD95L/FasL, and CD30) for most of the TNF-like receptors and ligands shown here to be expressed by resting or activated eosinophils [5, 8-10, 56-60]. Based on this evidence, it appears that eosinophils, following recruitment and activation by H-RS- and T-cell-derived cytokines (IL-5, IL-3, GM-CSF), may in turn interact with both these latter cell types in HD tissues. As a result of such interactions, eosinophils may contribute, along with membrane-bound ligands (CD30L, CD40L, FasL), surface receptors (CD40, CD95/Fas, 4-1BB, OX40), and soluble cytokines (TGF-β, GM-CSF, IL-1, IL-3, IL-5, IL-6 and IL-8 ) [23-26], to the deregulated cellular reaction underlying tumor cell growth and survival in HD [2-6, 8]. Whether eosinophil-derived CD40L, CD30L, and FasL may actually regulate H-RS cell growth in vivo remains to be established, but our in vitro data point in that direction.

The prognostic relevance of tissue eosinophil infiltration in HD appears controversial [14, 25, 61-65], even though recent results have suggested that it may represent a negative prognostic factor affecting disease-free survival [64]. Should this latter observation be confirmed by other clinical series, HD again will turn out to be a tumor with unique biologic properties. Usually a prominent eosinophilic infiltration within tumor tissues predicts a favorable outcome in other malignancies including cervix, lung, gastric, and colorectal carcinomas [25, 26].

Taken together, our data suggest that eosinophils may not merely be innocent bystanders but instead act as...
important elements in the pathology of HD by contributing to the deregulated network of interactive signals between H−RS cells and surrounding reactive cells. These interactions, mainly mediated by signaling of TNF-superfamily ligands through their specific counterreceptors on H−RS cells, such as CD40 [32, 35] and CD30 [65], may turn out to be of great relevance for the growth and survival of malignant cells in HD.

Acknowledgements

Supported in part by the Associazione Italiana per la Ricerca sul Cancro, Milan, Italy; the Ministero della Sanità, Ricerca Finalizzata IRCCS, Rome, Italy; the Consiglio Nazionale delle Ricerche, PF-ACRO (grant no. 92.02347.PF39), Italy; and the Deutsche Krebshilfe, Bonn, Germany.

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