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Thymus involvement in murine acquired immunodeficiency (MAIDS)

SONIA COLOMBI, MANUEL DEPREZ, LAURENCE DE LEVAL, CHANTAL HUMBLET, ROLAND GREIMERS, MARIE-PAULE DEFRESNE, JACQUES BONIVER & MICHEL MOUTSCHEN
Laboratory of Pathology, University of Liège, Liège, Belgium

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Abstract. Due to self-renewal of the peripheral pool of T-cells, adult thymectomy has normally little influence on immunocompetence. However, thymus might play a more important role in the setting of viral-induced cytopathic effects on T-cells in the periphery. Therefore, thymus weight, cell numbers, and subset distribution were sequentially analysed after infection with RadLV-Rs, a viral mixture known to induce murine retrovirus induced immunodeficiency (MAIDS). Infection induced thymic atrophy (concerning organ weight as well as total cell number) which culminated seven weeks after inoculation. The atrophic process mostly reflected the depletion of double positive $CD4^+$ $CD8^+$ cells since their proportion sharply decreased around week 6. Single positive T-cells were less affected by the process. The proportion of B-cells progressively increased. Surprisingly, there was a strong correlation between the extent of atrophy and the frequency of B-cells in the thymus. Finally, an abnormal $CD4^+$ T-cell subset lacking Thy-1 and previously described in the periphery also appeared in the thymus and its frequency was strongly correlated with the expansion of B-cells in this organ.

Abbreviations: DP – double positive; LN – lymph nodes; MAIDS – Murine Acquired Immunodeficiency Syndrome

Introduction

Immunodeficiency develops in mice infected with the RadLV-Rs (Duplan) strain of murine leukemia viruses (also called LP-BM5 MuLV). The syndrome is also characterized by lymphadenopathy and splenomegaly [1–3]. Both T-cells (especially of the $CD4^+$ subset) and B-cells [4] participate in the expansion of the lymphoid pool and display abolished responses to mitogen stimulation in vitro. The exact pathogenesis of the syndrome is unknown but close

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interactions between T-cells and B-cells must occur, possibly through the expression of a viral superantigen by the latter subset [5, 6]. Immunodeviation from secretion of T_{H1} to T_{H2} cytokines by $CD4^+$ T-cells has also been described [7]. Although MAIDS is clearly distinct from AIDS, similar immunological abnormalities have been pointed out in both infections [8].

The normal life-span of mature T-cells is supposed to be short [9, 10]. Therefore, their number must be maintained either by self-renewal of the peripheral pool or by differentiation taking place in the thymus. Population dynamics experiments conducted in thymectomized animals have revealed that the maintenance of the peripheral naive pool (i.e. virgin of any supraliminar stimulation of TCR by nominal antigen) relied mostly upon replenishment from the thymus. Although adult thymectomy has normally little impact on immunocompetence, the impairment of thymus function could have dramatic consequences in the setting of viral-induced cytopathic effects on peripheral T-cells (such as in HIV infection). It could also allow the progressive disappearance of the peripheral naive T-cells if a polyclonal stimulus (i.e. a superantigen) simultaneously induces a massive shift towards the memory/activated phenotype. Such a situation could occur in MAIDS since T-cells with a naive phenotype (especially within the $CD8^+$ subset) are found to be decreased late in the disease [unpublished observations]. In addition to the analysis of its contribution to peripheral immunodeficiency, there is also an interest to determine if thymus is susceptible to the processes taking place in the lymph nodes and spleen with regard to the low frequency of B-cells and the immaturity of T-cells in the former organ.

In this context, our study proposes to sequentially analyse thymus cellularity and subset distribution in the course of RadLV-Rs infection. An emphasis is made on phenotypic abnormalities previously described in the peripheral lymphoid organs: the appearance of $CD4^+$ T-cell lacking Thy-1 [11, 12] and high membrane density expression of CD44 [13].

Materials and methods

Mice and cell suspensions. Male C57Bl/Ka (H-2b) mice were bred in our facility. Mice were injected twice i.p. at the age of 4 and 5 weeks with 0.25 ml RadLV Duplan MulV stock solution. Age-matched control mice were injected twice with 0.25 ml saline. After different time intervals (first injection = time 0), mice were sacrificed by CO_2 asphyxiation. Thymus and LN were removed and weighed on a precision scale. Single cell suspensions were separately prepared with a fitting glass homogeneizer, passed through a nylon cell strainer, washed three times and counted on a Thoma hemacytometer.

Virus. Extract was prepared from the lymph nodes and spleen from three mice injected two months previously with RadLV-Rs extract 64. RadLV-Rs extract 64 was kindly provided by E. Legrand (INSERM 117, Bordeaux, France) and was described previously [1]. Lymphoid organs were ground in PBS and centrifuged 30 min at 15,000 g. The supernatant was spun again for 30 min at 15,000 g. This cell-free supernatant constituted the extract. It was immediately injected into mice or stored in liquid nitrogen. XC plaque assay [14] was used to measure virus titre. The virus preparation contained 1.0×10^3 PFU of ecotropic virus/ml.

Antibodies. The mAb used were fluorescein isothiocyanate (FITC) conjugated anti-Thy1.2 (30-H12), FITC-conjugated anti-CD8 (53-6.7), phycoerythrin (R-PE)-labelled anti-CD4/L3T4 (GK1.5) (Becton Dickinson, Erembodegem, Belgium), biotinylated anti-mouse CD44 (IM7) (Pharmingen), anti-CD3 mAb (145-2C11) (provided by Dr. J.A. Bluestone, University of Chicago, Chicago, IL) and PE-conjugated anti-B220 (6B2) (Gibco BRL). Purified anti-FcγRII (CD32) (2.4G2) was purchased from Pharmingen (San Diego, CA). 145-2C11 was conjugated to FITC or biotin following published protocols. Streptavidin (SA)-PE and streptavidin-FITC (Dako or Gibco BRL) were used as a second step reagent to reveal biotinylated antibodies.

Flow cytometry. Single cell suspensions were prepared from thymus and stained with optimal amounts of mAb on ice for 20 min in PBS with 2% BSA and 0.1% sodium azide. Cells were washed twice and counterstained with streptavidin-PE or streptavidin-FITC. After additional washes, cells were analysed on a FACStar Plus® (Becton Dickinson). Gating was performed according to forward and side scatter histograms. Data were collected and processed using the LYSIS II software (Becton Dickinson). Anti-Thy1.2 (30-H12) induced significant doublet formation between Thy-1^+ T-cells and B-cells or macrophages from infected mice. Therefore, cell suspensions were preincubated with anti-FcγRII (CD32) (clone 2.4G2) before the addition of anti-Thy1.2 (30-H12). The complete absence of doublets after this treatment was confirmed by FSC analysis and fluorescence microscopy.

Statistical analysis. Statistical analysis was performed using the Instat 2.01® software. According to the type of distribution, Student or alternate Welch t-tests were used. For non-gaussian distributions, the Mann-Whitney test was used. In all cases, two-tailed p values were considered. One asterisk (*) symbolizes p less than 0.05 and two asterisks (**) symbolize p less than 0.01. A logarithmic transformation was performed to normalize the data distribution in regression analysis.

Results

RadLV-Rs infection induces thymic atrophy. Sham-inoculation with PBS induced a moderate decrease of thymus weight and cellularity (not shown). In comparison, viral inoculation was associated with a more intense atrophy. Total cell number in the thymus of the infected mice was between 60 and 80% of controls until week 6 and strikingly decreased afterwards (Fig. 1). In contrast, the lymph node cells of the infected mice displayed a major expansion and reached 600% of control values at week 6 (Fig. 1).

MAIDS is associated with a late depletion of double positive thymocytes. There was no major alteration in the distribution of thymocyte subsets in the early stages of the infection and the proportion of $CD4^+$ $CD8^+$ (DP) remained constant until week 5 post-inoculation. Afterwards, a significant depletion of this subset was observed and became very significant at late time points (Fig. 2). At week 8, $CD4^+$ $CD8^+$ cells represented less than 40% of thymus cells in the infected animals (versus 80% in controls). Considering the important thymic atrophy occurring at that time of the infection, this important decrease of the proportion of $CD4^+$ $CD8^+$ cells actually corresponded to a major depletion of this subset (around 85% depletion at week 8).

Viral infection increases the frequency of T-cells with a mature phenotype. Consistent with the decreased frequency of DP cells in the thymus of the infected mice, the proportion of single positive $CD4^+$ and $CD8^+$ cells was found to be increased. For example, at week 8 post-inoculation, single positive $CD4^+$ cells represented around 40% (versus less than 10% in control animals) while single positive $CD8^+$ cells were around 15% (versus less than 5% in controls) (Fig. 3). In view of the decreased cellularity of thymus in the infected animals, this relative increase of single positive subsets actually corresponded to a moderate reduction of their absolute number (not shown).

There was a parallel increase of $CD4^+$ and $CD8^+$ T-cells with a high expression of CD3 (Fig. 4). Although triple staining was not performed in this study, the excellent correlation existing between the frequency of single positive cells and the frequency of $CD3^{hi}$ cells (not shown) suggested that most single positive T-cells observed in the thymus of the infected mice were $CD3^{hi}$.

Although MAIDS is associated with the peripheral expansion of $CD4^+$ and $CD8^+$ T-cells with a memory/activated $CD44^{hi}$ phenotype, there was only a weak increase of the proportion of these cells in the thymus (Fig. 3). Interestingly, in comparing the evolution of single positive versus $CD44^{hi}$ T-cells within $CD4^+$ and $CD8^+$ subsets, it appears that a large proportion of single positive $CD4^+$ cells has to be $CD44^{lo}$ whereas most single positive $CD8^+$ T-

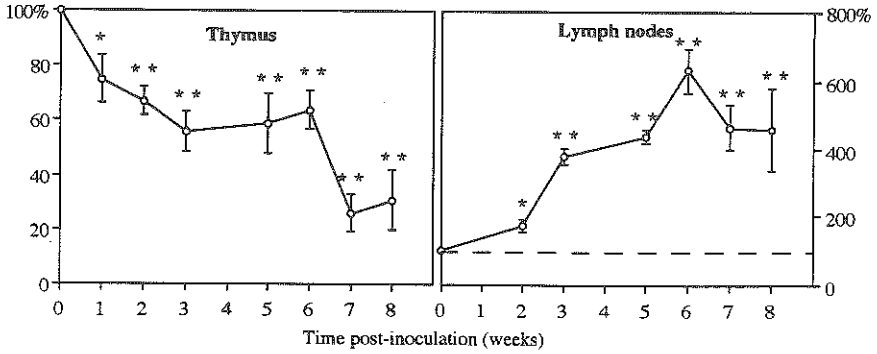

Fig. 1. Total cell numbers in the thymus (left panel) and lymph nodes (right panel) of infected mice. The values are given in percents of control values (mean of three sham-injected control mice) and represent means ± standard errors on the mean (sem) from groups of seven mice analysed individually. In both organs, the experimental values are significantly ($p < 0.05$) different from 100% on week 1 and very significantly ($p < 0.01$) different from 100% afterwards. The scale of the y-axis is different in each panel.

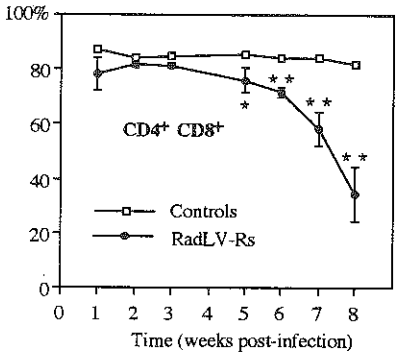

Fig. 2. Proportion of double positive (DP) CD4+ CD8+ T-cells in the thymus of RadLV-Rs infected mice (plain circles) and sham-injected controls (white squares). Double staining was performed using PE-conjugated anti-CD4 mAb and FITC-conjugated anti-CD8 mAb. The values represent means ± sem from groups of three controls and seven infected mice.

Viral infection increases the frequency of B-cells and CD4+ Thy-1−-cells. MAIDS is associated with a polyclonal expansion of B-cells in the peripheral lymphoid organs. We previously showed that, in thymus and LN of the infected mice as well as in controls, B220+ cells are mostly negative for T-cell markers such as CD4, CD8 and CD3 (not shown). Therefore, B220 remains a specific marker for the analysis of B-cells in MAIDS. This marker was consequently used to examine the evolution of B-cells in the thymus of the infected animals. The proportion of B220+ cells in control thymuses was less than 1%.

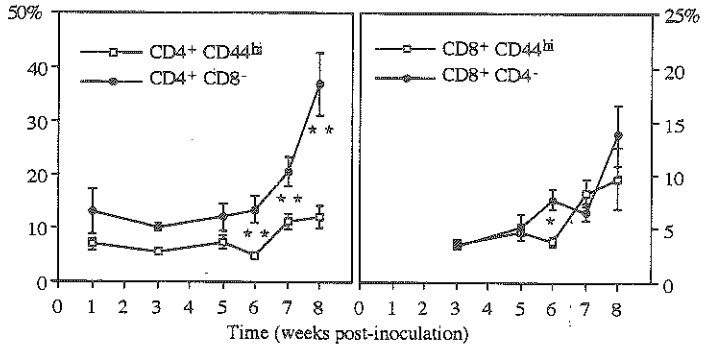

Fig. 3. Proportion of CD4$^+$ (left panel) and CD8$^+$ (right panel) T-cells characterized by single positivity (plain circles) and high expression of CD44 (white squares) in the thymus of infected animals. Double staining was performed with PE-labelled anti-CD4, FITC-labelled anti-CD8 and streptavidin-conjugated anti-CD44. FITC- or PE-conjugated avidin was used to reveal the anti-CD44 antibody. The values are the proportions of the given subsets (i.e. CD4$^+$ CD44^{hi}) within the total thymus population and represent means ± sem from groups of seven infected mice analysed individually. No variation was observed in the thymus of control mice (i.e. at week 8, the proportion of CD4$^+$ CD44^{hi} T-cells in the thymus of control mice was 5.57 ± 0.72% versus 12.19 ± 2.14% in infected animals). The scale of the y-axis is different in each panel.

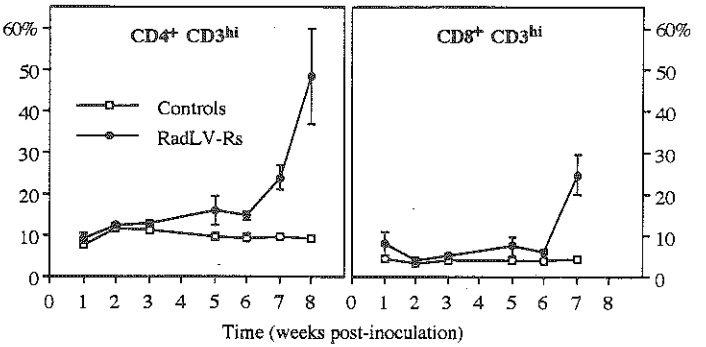

Fig. 4. Proportion of CD4$^+$ (left panel) and CD8$^+$ (right panel) T-cells characterized by a high expression of CD3 in the thymus of infected mice (plain circles) and sham-injected controls (open squares). Double staining was performed with FITC-labelled anti-CD8 mAb, PE-labelled anti-CD4 mAb and SA-conjugated anti-CD3 mAb. FITC or PE-conjugated avidin were used to reveal the anti-CD3 antibody. The values are the proportions of the given subsets (i.e. CD4$^+$ CD3^{hi}) within the total thymus population and represent means ± sem from groups of three controls and seven infected animals analysed individually.

of the infection. At week 5, a moderate but significant increase was observed (not shown). At week 7, the frequency of thymic B220$^+$ cells dramatically increased in the infected mice (Fig. 5) with an average value around 4.0% (not

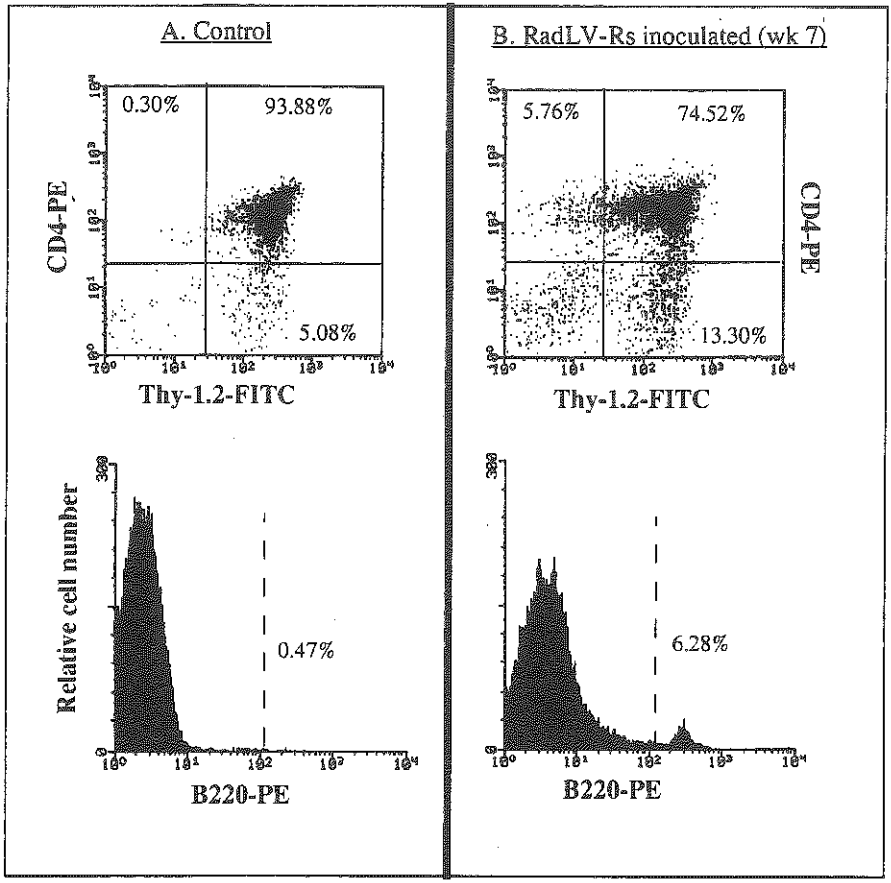

Fig. 5. Presence of B-cells (histograms) and CD4$^+$ T-cells lacking Thy-1 (dot plots) in the thymus of a RadLV-Rs infected mouse at week 7 postinoculation (B. right panel) and a sham-injected control (A. left panel). B-cells were stained with PE-conjugated anti-B220 mAb. Double staining was performed with FITC-conjugated anti-Thy-1.2 and PE-conjugated anti-CD4 mAb. In the control mouse, CD4$^+$ Thy-1$^-$ cells represent 0.32% of the total number of CD4$^+$ thymocytes while they represent 7.17% of CD4$^+$ thymocytes in the infected mouse.

increase at later time points (not shown). Although all infected animals displayed an increased frequency of thymic B220$^+$ cells, the extent of the maximal increase was highly variable from one animal to the other (not shown). Infection with RadLV-Rs was also responsible for the appearance in the thymus of an abnormal subset of CD4 T-cells lacking Thy-1 (Fig. 5). This expansion occurred with an important delay compared to lymph nodes and spleen cells (not shown).

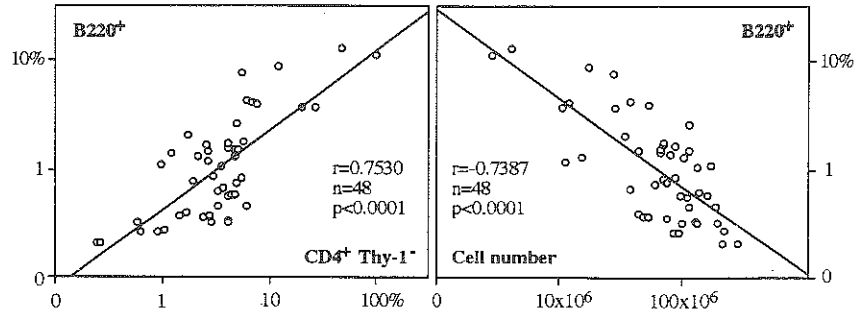

Fig. 6. The frequency of B-cells in the thymus of infected mice is negatively correlated with cellularity (right panel) and positively correlated with the frequency of CD4+ T-cells lacking Thy-1 (left panel). Phenotypic analysis was performed as described above. Individual values from week 1 to week 8 post-inoculation were logarithmically transformed to normalize their distribution and plotted using the Cricketgraph® software.

The frequency of B-cells in the thymus of the infected mice is correlated with thymic atrophy and with the frequency of CD4+ lacking Thy-1. We used the interindividual variability described above to determine if some of the abnormalities could be correlated. Interestingly, the frequency of B-cells was negatively correlated with the total cell number of the thymus (Fig. 6). There was also a strong positive correlation between the frequency of B-cells and the proportion of CD4+ T-cells lacking Thy-1 (Fig. 6).

Discussion

In this report, we provided new information concerning thymus in murine retrovirus-induced immunodeficiency: (1) Infection is associated with thymic atrophy occurring in two phases (moderate between week 1 and week 6 and more pronounced afterwards); (2) The atrophy mostly reflects the dramatic depletion of double positive CD4+ CD8+ cells occurring around week 6 post-inoculation; (3) The number of single positive cells is far less affected by infection and their relative proportion increases; (4) An important part of the single positive CD4+ cells has a low expression of CD44 and could therefore originate from in situ differentiation rather than from the periphery where most CD4+ T-cells have a memory/activated phenotype at this stage of the infection; (5) The frequency of B-cells increases in the thymus of the infected animals and is negatively correlated with cellularity. A positive correlation is also found with the frequency of an abnormal subset of CD4+ T-cells lacking Thy-1, previously described in the peripheral lymphoid organs.

It should be pointed out that despite the infection, the animals are still in good condition when major thymic atrophy occurs around week 6. Furthermore, the participation of stress hormones induced by injections or acute infection is unlikely in view of the comparison with sham-injected controls and of the relatively late occurrence of major atrophy. Although we cannot formally rule out a participation of the stress associated with chronic or subacute viral infection, we think that the depletion of DP thymocytes is largely due to factors specific to RadLV-Rs infection since this depletion is closely correlated with other typical manifestations of MAIDS such as the appearance of $CD4^+$ T-cells lacking Thy-1 and the expansion of $B220^+$ cells.

The depletion of DP $CD4^+$ $CD8^+$ cells could result either from cytopathic effects of the virus or alternately from an accelerated differentiation process with rapid migration of the mature single positive cells to the periphery. Previous reports have indicated that the defective retrovirus infects mostly B-cells rather than T-cells [15]. Although this fact has to be formally proved in the thymus, it is unlikely that the depletion of DP $CD4^+$ $CD8^+$ T-cells could be due to intracellular infection by the defective virus. Alternately, interactions of DP cells with infected B-cells could be responsible for their depletion. Soluble factors (such as IL-10 secreted by the activated B-cells) or direct intercellular contacts could take part in this interaction. A putative TCR-ligand with superantigen-like properties has been described in B-cell lines derived from mice infected with LP-BM5 MuLV [5, 6]. Polyclonal stimulation of DP thymocytes by such a superantigen could therefore induce massive apoptosis within this subset. Work is in progress in our laboratory to objectivate the presence of apoptosis in the thymus of infected mice.

Since the effects of superantigens are dependent on the presence of MHC class-II products, the interaction with infected B-cells could skew the differentiation of DP thymocytes towards single positive $CD4^+$ $CD8^-$ T-cells and explain the presence of an important proportion of $CD4^+$ $CD8^-$ $CD44^{lo}$ cells in the thymus of the infected animals. This could also contribute to the major increase of the CD4/CD8 ratio observed in the peripheral lymphoid organs within the memory as well as in the naive subsets [unpublished observations].

Both T-cells [16] and B-cells [17] are necessary to sustain the polyclonal proliferation observed in MAIDS. Therefore, B-cells in the thymus must receive some signals from local T-cells to proliferate. The relatively weak expansion of B-cells in the thymus compared to periphery could therefore be due to the low frequency of T-cells capable of delivering appropriate signals sustaining their proliferation. However, it is important to note that even at late time points when most T-cells in the thymus have a mature phenotype, this organ does not participate in the lymphoproliferative process which involves LN, spleen and non lymphoid organs such as liver and lungs. This absence of lym-

from the periphery migrate to the thymus, their proliferation must be inhibited locally by some undetermined factor.

We propose a hypothesis in which most abnormalities of thymic subsets occurring in MAIDS would result from local interactions of thymocytes with infected B-cells. Due to their immaturity, T-cells from the thymus would respond to this interaction by programmed cell death instead of proliferation and generation of B-cell help. Work is in progress to formally prove this hypothesis.

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Address for correspondence: Michel Moutschen, MD, Département de Pathologie, CHU du Sart-Tilman, B-4000 Liège, Belgium
Phone: (32) 41 56 2 410; Fax: (32) 41 56 2 919