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Cutting Edge: Fasting-Induced Hypoleptinemia Expands Functional Regulatory T Cells in Systemic Lupus Erythematous

Yaoyang Liu,*† Yiyun Yu,* Giuseppe Matarese,*‡ and Antonio La Cava*

Fasting is beneficial in the prevention and amelioration of the clinical manifestations of autoimmune diseases including systemic lupus erythematosus. The mechanisms responsible for these effects are not well understood. During fasting, there is a dramatic reduction of the levels of circulating leptin, an adipokine with proinflammatory effects. Leptin also inhibits CD4+CD25+Foxp3+ regulatory T cells, which are known to contribute significantly to the mechanisms of peripheral immune tolerance. In this study, we show that fasting-induced hypoleptinemia in (NZB × NZW)F1 lupus-prone mice induced an expansion of functional regulatory T cells that was reversed by leptin replacement. The specificity of the findings was indicated by the lack of these effects in leptin-deficient ob/ob mice and leptin receptor-deficient db/db mice. These observations help to explain the beneficial effects of fasting in autoimmunity and could be exploited for leptin-based immune intervention in systemic lupus erythematous. The Journal of Immunology, 2012, 188: 2070–2073.

During fasting, the levels of the circulating adipokine leptin are dramatically reduced (5). Leptin has all of the characteristics of a proinflammatory cytokine (6) and links nutritional status with neuroendocrine functions and immune responses. Leptin promotes Th1 cell differentiation and the development of autoimmune responses in several animal disease models (7), and it can act as a negative factor for the expansion of CD4+ regulatory T cells (TReg), a subset of T cells with an important role in the maintenance of peripheral immune tolerance to self-Ags (8).

In an attempt to connect these observations, we investigated the effects of fasting in NZB/W mice and found that the reduction in circulating levels of leptin caused by starvation directly promoted the expansion of functional TReg. These findings can help to explain some of the beneficial effects of fasting in SLE.

Materials and Methods

Mice

C57BL6/J (B6) wild-type (WT) mice, leptin-deficient B6ob/ob (ob/ob), leptin receptor-deficient B6db/db (db/db), and NZB/W mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the University of California Los Angeles. Mice were treated in accordance with institutional guidelines under approved protocols. All experiments were conducted in age-matched female mice that were divided into three groups. One control group had ad libitum access to food and received i.p. injections of 0.2 ml PBS at 9 AM and 6 PM daily for 2 d. The other two groups of mice were deprived of food for 48 h and received i.p. injections of either 0.2 ml PBS or recombined leptin (R&D Systems, Minneapolis, MN) dissolved in PBS at a dose of 1 μg/g body weight twice daily (also at 9 AM and 6 PM). All mice had continuous access to water.

Cell isolation and staining

After blood drawing, erythrocytes were removed using red cell lysing buffer (Sigma-Aldrich, St. Louis, MO), and PBMC were used for flow cytometry. For splenocytes, single-cell suspensions were prepared by passing cells through a cell strainer before red cell lysis and resuspension in HL-1 medium (BioWhittaker, Walkersville, MD). CD4+CD25+ and CD4+CD25− T cells were isolated via magnetic bead separation with Miltenyi Biotec kits using an AutoMACS System (Miltenyi Biotec, Auburn, CA) and found >95% pure by flow cytometry analysis.

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The online version of this article contains supplemental material.

Abbreviations used in this article: SLE, systemic lupus erythematosus; TReg, regulatory CD4+ T cells; WT, wild-type.

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Flow cytometry
Phenotypic analyses were performed with combinations of fluorochrome-conjugated Ab using standard techniques. After Fc blocking, fluorochrome-conjugated anti-mouse Ab to CD4 and CD25 (eBioscience, San Diego, CA) or isotype control Ab were used for staining prior to acquisition on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and subsequent analysis using FlowJo software (Tree Star, Ashland, OR). For intracellular staining, cells were first stained for the expression of cell-surface markers and then fixed, permeabilized, and stained using the Foxp3 staining kit (eBioscience) according to the manufacturer’s instructions.

Suppression assays
After cell isolation, CD4^+^CD25^-^ effector T cells (Teff) were labeled with CFSE in incubation by FACS at 37°C for 10 min. Samples were then placed in HL-1/5% FCS and incubated in ice for 5 min, then washed two times. Teff/Teff (ratio 1:1 or 1:4) mixtures or control samples consisting of Teff alone were plated in 96-well round-bottom plates (Corning Life Sciences, Lowell, MA). Dynabeads mouse anti-CD3/CD28 beads were added to a final ratio of 0.5 beads/cell. Samples were incubated in a 37°C incubator for 72 h. Proliferation of CFSE-labeled Teff was evaluated by flow cytometry and expressed as FlowJo software calculated division index (the average number of cell divisions). Supernatants were collected for ELISA detection of IFN-γ in the cocultures. Percent suppression was calculated using proliferation measurements and the following formula: percent suppression = (1 − Teff/Teff) × 100.

ELISA
Concentration of leptin and IFN-γ were determined by commercial ELISA kits (R&D Systems) according to the manufacturer’s instructions.

Statistical analyses
Analyses were performed using Prism 4 software (GraphPad, San Diego, CA). Nonparametric testing among three groups was performed by Kruskal-Wallis ANOVA. Comparisons among three groups were performed by Dunn’s multiple comparison test. The p values < 0.05 were considered statistically significant.

Results and Discussion
The effects of fasting were compared among WT mice, leptin-deficient ob/ob mice, leptin receptor-deficient db/db mice, and lupus prone NZB/W mice. Control animals were fed ad libitum or starved 48 h (treated or not with leptin). As expected, starvation led to a reduction in body weight in all groups of mice when compared with ad libitum-fed animals (Table I). Starvation also associated with a significant reduction in the number of splenocytes and Teff in starved WT, ob/ob, and NZB/W mice (Supplemental Table I).

It is well established that fasting associates with the reduction of circulating leptin levels (5). Accordingly, WT and NZB/W mice starved for 48 h had hypoleptinemia when compared with ad libitum-fed animals, whereas no changes in leptin levels were observed in starved ob/ob mice [which cannot produce functional leptin because of a mutation in the leptin gene (9)] and db/db mice [which carry a mutation in the leptin receptor (10)] (Table I). The administration of leptin to starved mice restored the circulating levels of this adipokine to normal concentrations in leptin-sufficient mice (Table I), and spleen cellularity returned to values similar to those seen in ad libitum-fed controls in starved mice that received leptin (Supplemental Table I).

Because we reported that leptin can constrain the expansion of TReg in vivo (8), we investigated whether the reduction in circulating levels of leptin induced by fasting could affect the TReg numbers. It was found that the frequency of TReg in starved WT and NZB/W lupus mice was significantly increased as compared with ad libitum-fed mice (Fig. 1). Of interest, leptin administration to ob/ob mice, which had no change of leptin levels after starvation (Table I), associated with a reduction in the number of TReg (Fig. 1C, 1D), suggesting that leptin per se has inhibitory effects on the expansion of TReg in vivo. Also, db/db mice (which are hyperleptinemic but unresponsive to leptin) showed no change in TReg frequency after starvation, and leptin replacement did not change this finding (Fig. 1E, 1F). The TReg that had expanded after starvation had similar CD25 expression (which can be a marker of immune cell activation) in the different groups of animals (Supplemental Table I), and the frequency of CD4^+^CD25^-^ cells (Teff) in PBMC was reduced by fasting and restored by leptin replacement in WT, ob/ob, and NZB/W mice (Supplemental Table I).

Taken together, these results indicate that fasting associates with an increase in TReg frequency when the leptin pathway is intact (in WT and NZB/W mice), and the TReg frequency remains unaltered when fasting cannot alter leptinemia due to an impairment of the leptin/leptin receptor axis (such as in ob/ob and in db/db mice). These events depend on leptin because in WT and NZB/W mice the TReg frequency returned to numbers comparable to those found in ad libitum-fed mice following leptin replacement (Fig. 1).

To also address whether the observed changes in TReg frequency associated or not with a normal function of the suppressor cells, in vitro suppression assays were performed. The

<table>
<thead>
<tr>
<th>Mice</th>
<th>Ad Libitum</th>
<th>Fast + PBS</th>
<th>Fast + Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Initial body weight (g)</td>
<td>20.59 ± 0.72</td>
<td>20.97 ± 0.79</td>
</tr>
<tr>
<td></td>
<td>Plasma leptin (ng/ml)</td>
<td>7.22 ± 0.67</td>
<td>4.06 ± 0.64**</td>
</tr>
<tr>
<td>ob/ob</td>
<td>Initial body weight (g)</td>
<td>58.36 ± 0.61</td>
<td>58.42 ± 1.39</td>
</tr>
<tr>
<td></td>
<td>Plasma leptin (ng/ml)</td>
<td>58.46 ± 0.60</td>
<td>54.82 ± 1.47</td>
</tr>
<tr>
<td>db/db</td>
<td>Initial body weight (g)</td>
<td>59.5 ± 0.29</td>
<td>7.01 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Plasma leptin (ng/ml)</td>
<td>59.6 ± 0.25</td>
<td>52.9 ± 2.89</td>
</tr>
<tr>
<td>NZB/W</td>
<td>Initial body weight (g)</td>
<td>9.43 ± 1.13</td>
<td>8.01 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>Plasma leptin (ng/ml)</td>
<td>9.28 ± 0.45</td>
<td>23.63 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>Body weight after 48 h (g)</td>
<td>23.87 ± 0.42</td>
<td>19.92 ± 0.58****</td>
</tr>
<tr>
<td></td>
<td>Plasma leptin (ng/ml)</td>
<td>9.79 ± 0.40</td>
<td>5.75 ± 0.48****</td>
</tr>
</tbody>
</table>

Mice were fed ad libitum or fasted 48 h. Values represent mean ± SEM.

*p < 0.05 versus initial body weight; **p < 0.05 versus ad libitum; ***p < 0.05 versus fast + PBS; ****p < 0.01 versus ad libitum and fast + PBS; *****p < 0.001 versus initial body weight; ******p < 0.01 versus ad libitum.
results showed that the changes in T_{Reg} frequency under the different conditions (ad libitum, fast, and fast + leptin) did not associate with differences in the suppressive capacity of T_{Reg} on T_{Eff} (Fig. 2, Supplemental Table II), indicating that T_{Reg} expanded by fasting were functionally comparable among groups.

Altogether, these results widen earlier observations that showed that caloric restriction ameliorated SLE manifestations (2, 11, 12). The observation that functional T_{Reg} could expand during fasting in lupus mice is relevant to the disease pathogenesis because the frequency of T_{Reg} is lower in NZB/W mice as compared with WT mice (13, 14), and this aspect has been linked to an inability of the dysfunctional immune system in SLE to control disease course and progression (15).

The link between nutritional status and immune regulation suggested by recent reports that showed that the mammalian target of rapamycin pathway could influence the activity of T_{Reg} through leptin (16) identifies in the current study a new possibility to modulate T_{Reg} activity in SLE via leptin-based intervention [also considering that leptin is elevated in SLE patients (17)]. In particular, the frequency of T_{Reg} could be tuned for therapeutic purposes in SLE not only through fasting but also, more specifically, using neutralizing Abs to leptin to promote the expansion in vivo of functional T_{Reg} in the disease.

Disclosures
The authors have no financial conflicts of interest.
FIGURE 2. In vitro suppression of T_{Eff} proliferation by T_{Reg} (1:1 ratio) in ad libitum-fed versus 48 h-starved mice without or with leptin replacement. (A) WT mice; (B) ob/ob; (C) db/db, and (D) NZB/W mice. The figure shows proliferation in flow cytometry of anti-CD3/CD28 Ab-stimulated, splenocyte-derived, CFSE-labeled T_{Eff} cultured alone (gray line) or with (black line) T_{Reg}.

References