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Anti-CD40L Accelerates Renal Disease and Adenopathy in MRL-\textit{lpr} Mice in Parallel with Decreased Thymocyte Apoptosis\textsuperscript{1}

Jennifer Q. Russell,\textsuperscript{*} Thomas Mooney,\textsuperscript{*} Philip L. Cohen,\textsuperscript{‡} Bruce MacPherson,\textsuperscript{‡} Randolph J. Noelle,\textsuperscript{§} and Ralph C. Budd\textsuperscript{2,*}

The CD40/CD40L (CD40 ligand) axis regulates several interactions between T cells and B cells. Blocking of CD40 engagement by CD40L inhibits Ig class switch by B cells as well as diminishes T cell response to an immunizing Ag. For these reasons, disruption of CD40/CD40L interactions by anti-CD40L administration or by genetic disruption of CD40L has ameliorated a variety of autoimmune conditions. More recent findings suggest that a direct signal can be transmitted to T cells via their expressed CD40L, which can costimulate proliferation with CD3 or promote germinal center formation. It is therefore possible that treatment with anti-CD40L Ab might produce a different outcome than observed in genetically CD40L-deficient mice. In this regard, we observe that in contrast to the genetic deletion of CD40L in MRL-\textit{lpr} mice, which diminishes autoimmune disease but has little effect on adenopathy, administration of anti-CD40L to MRL-\textit{lpr} mice accelerates both of these parameters. This difference appears to result from anti-CD40L actively delivering a signal that inhibits T cell apoptosis in \textit{lpr} mice. This was confirmed by in vitro studies demonstrating that CD40L cross-linking on \textit{lpr} thymocytes inhibited apoptosis and surface TCR down-modulation induced by CD3 ligation. The \textit{Journal of Immunology}, 1998, 161: 729–739.
The latter is due, not to increased proliferation of lymphoid cells, but rather to their decreased rate of apoptosis. The findings suggest that CD40L can mitigate apoptotic signals in T cells.

Materials and Methods

Mice

MRL-\(lpr/lpr\) mice were bred in the animal facilities at The University of Vermont College of Medicine from original breeding pairs obtained from The Jackson Laboratory (Bar Harbor, ME).

Anti-CD40L treatment

Eight-wk-old female MRL-\(lpr/lpr\) mice received either hamster monoclonal IgG anti-murine CD40L, MR1 (33), or control hamster IgG (ICN Pharmaceuticals, Costa Mesa, CA) at 250 \(\mu\)g i.p. twice weekly for 3 wk. In experiment 1, eight mice were treated per group, and one mouse from each group was euthanized after weeks 4 and 5 for analysis of lymphoid tissues. In experiment 2, 12 mice were treated in each group, and one mouse from each group was euthanized at weeks 2.5, 4, and 5 for analysis of lymphoid tissues and renal histology. The remaining six mice in experiment 1, and nine mice in experiment 2, were monitored for survival and quantitation of proteinuria using Chemstrip (Boehringer Mannheim Diagnostics, Indianapolis, IN) over an 18-wk (Expt. 1) or 20-wk (Expt. 2) period. Mice that achieved maximal 3+ proteinuria and weight loss were considered terminated.

Kidney histology

Fresh tissues were fixed in buffered 10% (v/v) paraformaldehyde for 24 h, washed in 70% (v/v) ethanol, and embedded in paraffin blocks. Serial kidney tissue sections were cut, fixed on slides, and stained with either hematoxylin and eosin (H & E) or periodic acid-Schiff (PAS) to assess, respectively, renal pathology. Sections were scored in a blinded manner by a renal pathologist (B.M.P.). A 0 to 2+ scale was used to quantitate severity for glomerular cellularity, interstitial inflammation, nuclear debris, tuft necrosis, basement membrane thickening, and sclerosis. Scoring was performed on 40 glomeruli from each mouse (20 glomeruli in each of two sections). In addition, the number of glomerular nuclei were counted in 10 separate glomeruli from each section stained with either H & E or PAS.

Quantification of serum Igs and autoantibody levels by ELISA

Sera from representative mice in both experiments were taken at the times indicated and analyzed for levels of total IgG, IgG1, and IgM. In addition, serum levels of total IgG and IgG1 autoantibodies to ssDNA, Sm Ag, and chromatin, as well as IgM rheumatoid factor, were determined by ELISA as previously described (34).

Abs and flow cytometry

Monoclonal anti-murine CD8\(\alpha\) conjugated to phycoerythrin was purchased from Caltag Labs (Burlington, CA). Monoclonal anti-murine CD4 conjugated to Red613 was purchased from Life Technologies (Gaithersburg, MD). mAb to mouse TCR-\(\alpha\beta\), clone H57-597 (35), was purified from mouse ascites on HiTRAP protein G columns (Pharma Biotech, Piscataway, NJ) and then conjugated to fluorescein (Sigma Chemical, St. Louis, MO) using established methods (36). Fluorescein-conjugated Abs was purified from reaction components by chromatography on PD-10 columns (Pharma Biotech). Biotinylated anti-\(\alpha\)-chain mAb, clone 187-1, was the kind gift of Dr. Karen Newell (The University of Vermont, Burlington, VT).

Single cell suspensions were made by homogenizing tissues in RPMI 1640 medium (Life Technologies) supplemented with 5% (v/v) bovine calf serum (HyClone Laboratories, Ogden, UT). Cells excluding trypan blue were counted. For flow cytometry, 10⁶ cells were incubated in 0.1 ml PBS containing 0.5% BSA Fraction V, 0.001% (w/v) sodium azide (Sigma), and the indicated Abs, each at 3 \(\mu\)g/ml, at 4°C for 30 min. After washing with PBS-azide, cells were fixed in 1% (v/v) methanol-free formaldehyde (Ted Pella, Reading, CA) in PBS-azide. Samples were stored at 4°C until analysis with a Coulter Elite flow cytometer calibrated using DNA check beads (Coulter, Hialeah, FL). Data were gated using Elite software by forward and side light scatter. Negative controls were set by using isotype-matched Ig directly conjugated to fluorochromes (Caltag). To measure the surface expression of CD40L using MR1 mAb, thymocytes and LN cells were analyzed either when freshly isolated or after 4-h stimulation at 37°C in medium containing PMA (10 ng/ml) plus ionomycin (250 \(\mu\)g/ml).

TUNEL assay for apoptosis

Cells were initially stained for expression of TCR-\(\alpha\beta\), CD4, and CD8 and then fixed for 15 min in 1% formaldehyde. Cell membranes were then permeabilized for 15 min using 70% ethanol at 4°C. Samples were incubated at 37°C for 1 h in 50 \(\mu\)l containing 10 U terminal deoxynucleotidytransferase and 0.5 nM d-UTP-biotin (Boehringer Mannheim) (37, 38). Specimens were washed twice with PBS/1%BSA and incubated with a 1:50 dilution of streptavidin-tricolor (Caltag) at 4°C for 30 min. Cells were washed twice and analyzed by flow cytometry. Negative controls consisted of staining of cells with the same protocol but in the absence of d-UTP-biotin. Positive control staining for apoptosis was determined using thymocytes from mice that received 2 mg i.p. of dexamethasone 18 h previously.

In vitro anti-CD40L ligation of thymocytes

Single cell suspensions of thymocytes from 10-wk-old MRL-\(lpr/lpr\) mice were placed in RPMI 1640/5%FCS. Activation conditions included either control hamster IgG or anti-CD40L (10 \(\mu\)g/ml) for 20 min followed by cross-linking with goat anti-hamster IgG (50 \(\mu\)g/ml, Caltag) for 20 min and finally addition of anti-CD3 (500A2, 10 \(\mu\)g/ml). Timing was begun at the addition of anti-CD3, and cells were analyzed either freshly isolated or 3.5 h and 5.0 h after activation. Thymocytes were stained for expression of CD4, CD8, TCR-\(\alpha\beta\), fixed, and then stained by the TUNEL assay.

**FIGURE 1.** Anti-CD40L treatment of MRL-\(lpr/lpr\) mice accelerates the development of proteinuria (A) and diminishes survival (B). Six 8-wk-old female MRL-\(lpr/lpr\) mice per group received 250 \(\mu\)g i.p. of either hamster IgG (dashed line) or anti-CD40L Ab (solid line) twice weekly for a total period of 18 wk. Statistical analysis was by Wilcoxon signed rank test for proteinuria and log rank survival for the survival studies. Similar results were observed in a second 20-wk study using nine MRL-\(lpr/lpr\) mice per group.
Results

Anti-CD40L Ab treatment of MRL-Ipr mice accelerates glomerulonephritis and decreases survival

Given the existence of a wide variety of IgG autoantibodies in MRL-Ipr mice, we attempted to diminish their serum levels using administration of anti-CD40L in vivo, while monitoring proteinuria and survival. Details of the five separate experiments are given in the Materials and Methods. In brief, the first four experiments consisted of 3 wk of twice weekly anti-CD40L administration followed by a total 18-wk (Expt. 1) or 20-wk (Expt. 2) period of monitoring. A fifth experiment examined whether prolonged (15 wk) anti-CD40L treatment might more efficiently suppress IgG autoantibody formation.

In striking contrast to the findings in the (SWR × NZB)F<sub>1</sub> mice (25), as well as a recent report of diminished disease in CD40L-deficient/Ipr mice (32), anti-CD40L administration to MRL-Ipr mice resulted in accelerated disease and adenopathy, as well as decreased survival. The onset of proteinuria was earlier in MRL-Ipr mice receiving anti-CD40L. The first experiment is shown in Figure 1A and illustrates the percentage of mice that had achieved 3+ proteinuria during the 18-wk period. By 10 wk, four of the six mice (67%) receiving anti-CD40L had achieved 3+ proteinuria, whereas only two of six control mice that received hamster IgG (33%) manifested this degree of proteinuria. The increased proteinuria in the anti-CD40L group persisted throughout the 18-wk period of observation. The second experiment with 12 mice per group showed very similar accelerated proteinuria over a 20-wk period in the mice receiving anti-CD40L (data not shown). These differences were statistically significant in both experiments by both the Wilcoxon signed rank test ($p = 0.005$) and $t$ test ($p = 0.002$).

Survival was also diminished in the group of MRL-Ipr mice receiving anti-CD40L. As shown in Figure 1B, by the end of the 18-wk study period in experiment 1, only two of six mice were alive in the anti-CD40L treatment group, whereas all six mice that received hamster IgG survived. By log rank survival curve this was significant at $p = 0.019$. Similar findings, though somewhat less pronounced, were observed in the second experiment.

The more rapid onset of proteinuria in MRL-Ipr mice receiving anti-CD40L was reflected in histologic evidence of renal injury, as defined by the number of glomerular nuclei (Fig. 2A), as well as increased glomerular cellularity (Fig. 2B) and glomerular inflammation, with or without nuclear debris (Fig. 2C). The glomerular histology was assessed in a blinded manner by a renal pathologist. A statistically significant increase in the number of glomerular nuclei was observed at all three time points (2.5 wk, $p = 0.019$; 4 wk, $p = 0.043$; and 5 wk, $p = 0.005$ by $t$ test) in the mice receiving anti-CD40L, while a significant increase in the severity of glomerular cellularity and inflammation was seen in the same mice at 2.5 wk ($p = 0.001$ and 0.001, respectively) and 5 wk ($p = 0.001$ and 0.002, respectively). These findings correlate well with the accelerated proteinuria that was observed with anti-CD40L administration.

Anti-CD40L treatment does not significantly alter autoantibody production in MRL-Ipr mice

Since CD40 is required for Ig class switching from IgM to IgG, mice or humans deficient in CD40L manifest greatly diminished serum levels of IgG and frequently elevated levels of IgM (13, 18). In experiments 1 and 2, as MRL-Ipr mice received Ab during only the first 3 wk of the study, it is perhaps not surprising that serum levels of Ig isotypes were largely comparable between the two treatment groups, as were levels of IgG and IgM autoantibodies to ssDNA, Sm Ag, chromatin, and rheumatoid factor (data not shown). However, even with anti-CD40L treatment for up to 12 wk, as in experiment 5, there was still no consistent statistically significant difference in total serum IgG1 or IgM (Fig. 3, A and B). In a similar manner, although serum levels of IgG1 (Fig. 3, D–F), IgG2a, and IgG3 (data not shown) autoantibodies to ssDNA and

![FIGURE 2. Increased glomerular injury in MRL-Ipr mice receiving anti-CD40L. In Expt. 2, beginning at 8 wk of age, 12 female MRL-Ipr mice received 3 wk of biweekly hamster IgG (HIGG) or anti-CD40L, and one mouse per group was euthanized at weeks 2.5, 4, and 5. Both kidneys of each mouse were removed and fixed, and sections were stained with H&E or PAS. A, Absolute counts and means (horizontal bar) of number of nuclei for each of 10 glomeruli for each mouse. B, C, Mean ($±$ SEM) score of 40 glomeruli of each mouse analyzed for (B) increase in glomerular cellularity, or (C) severity of glomerular inflammation using a 0 to 2+ scale of severity. Statistical significance by $p$ value ($t$ test) is shown above each time point.](http://www.jimmunol.org/)
Sm Ag, but not chromatin, were slightly decreased in mice receiving anti-CD40L and the corresponding IgM autoantibodies somewhat increased (Fig. 3G), these did not achieve statistical significance. This suggests that the CD40/CD40L axis may have little impact on the development of several types of autoantibodies in MRL-1pr mice. This is in agreement with CD40L-deficient/1pr mice, which also were reported to manifest unaltered levels of certain autoantibodies (32).

Anti-CD40L treatment promotes adenopathy and the expansion of TCR-αβ+ cells in the thymus and LN of MRL-lpr mice

A remarkable expansion of T cell numbers occurred rapidly in both the thymus and LN during the first few weeks of anti-CD40L treatment, as is summarized for experiments 1 and 2 in Table I. This was highly statistically significant for both the thymus (p = 0.005) and LN (p = 0.007). As shown in Figure 4 (bottom row), as early as 2.5 wk after initiating anti-CD40L administration, thymus cellularity was considerably increased by 1.5- to 2-fold, and this persisted at 5 wk, at a point when anti-CD40L treatment had ended 2 wk previously.

An analysis of the phenotypes of the thymocytes revealed a slight but consistently enhanced proportion of mature single positive TCR-αβ+ thymocytes with anti-CD40L treatment (Fig. 4, compare top two rows). Although the proportions of CD4+CD8+ and CD4+CD8- thymocytes were correspondingly somewhat diminished with anti-CD40L, due to the pronounced increased thymic cellularity, the absolute numbers in these subsets were nonetheless considerably increased. The suggestion that a larger proportion of mature thymocytes emerged with anti-CD40L treatment is supported by an increase in the expression of TCR-αβ among all thymocyte subsets (Fig. 4, middle row). A complete listing of thymocyte and LN cell numbers and subset percentages is shown for experiments 1 and 2 in Table I. The total number of thymocytes was statistically increased with anti-CD40L treatment (p = 0.005) as well as the percentage of CD4+CD8- thymocytes (p = 0.027), while the proportional increase in CD4+CD8+ thymocytes was just below significance (p = 0.064).

Cellularity differences were even more striking in the LN. Anti-CD40L treatment produced up to a fivefold increase in LN cell number by 5 wk (Fig. 5, bottom row). In contrast to the thymus where the proportions of mature CD4+ and CD8+ T cells were increased, the expanding LN cell population with anti-CD40L manifest decreased proportions (though increased absolute numbers) of mature T cells, due to the dilution by TCR-αβ+ CD4+CD8- cells (Fig. 5, middle row). Over the course of experiments 1 and 2, both the total number of LN cells (p = 0.007) and
Table I. Increased lymphoid cell number and peripheral TCR-αβ⁺ CD4⁻CD8⁻ cells in MRL-lpr mice receiving anti-CD40L

<table>
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<th>Expt</th>
<th>Treatment</th>
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*Eight-week-old female MRL-lpr mice received twice weekly injections of 250 μg i.p. of either hamster IgG (HlgG) or anti-CD40L. One mouse per group was euthanized at the times indicated (based on the start of treatment), and thymus and LN (combined total of two axillary and two inguinal nodes) were analyzed for expression of CD4, CD8, and TCR-αβ. Paired t test analysis of the two experiments shows a statistical increase in the absolute numbers of thymocytes (p = 0.005) and LN cells (p = 0.007), as well as the percentage of LN TCR-αβ⁺ CD4⁺CD8⁻ cells (p = 0.024). The percentage of mature single positive thymocytes was increased, which reached significance for the CD4⁺CD8⁻ subset (p = 0.027) but not for the CD4⁻CD8⁺ subset (p = 0.064).

FIGURE 4. Anti-CD40L increases thymus cellularity and promotes the appearance of TCR-αβ⁺ thymocytes in all subsets. Shown are FACS profiles of the thymuses from 8-wk-old female littermate MRL-lpr mice that had received either hamster IgG (upper row) or anti-CD40L (middle row) twice weekly for 2.5 wk. Percentages of thymocytes in each gate are indicated by the number inserts. Note the slightly increased proportion of mature single positive thymocytes as well as the increased percentage expressing TCR-αβ with anti-CD40L treatment. The bottom row illustrates thymocyte absolute numbers in the subsets indicated. Hatched bars represent cell numbers in mice that received control hamster IgG, whereas solid bars correspond to cell numbers in anti-CD40L-treated mice. These findings were highly consistent over the course of five separate experiments using twice weekly treatment for 3 wk and examining tissues at intervals ranging from 1 to 5 wk.
the proportion of TCR-αβ+ CD4+CD8- cells (p = 0.024) were statistically increased in the group that received anti-CD40L (Table I).

A similar though not quite as profound increase of splenic B cells was also observed with anti-CD40L treatment of lpr mice. As shown in Figure 6, both the proportion as well as absolute number of splenic B cells was increased. The increase in B cell number was not manifested as quickly as were the increases of thymocyte and LN T cell numbers. Whereas the changes in thymocyte and LN cellularity was apparent as early as 1 wk in some experiments, the increase in splenic B cells was not observed until at least wk 3 or 4 of anti-CD40L treatment. The effects of anti-CD40L on lpr adenopathy were not limited to the MRL background since similar accelerated adenopathy was observed in C57BL/6-lpr mice that received anti-CD40L biweekly for 5 wk (data not shown). Morever, these differences were not likely related to alterations in Ag presentation since surface levels of MHC class I, class II, B7-1, and B7-2 were identical in the two groups of mice (data not shown).

Anti-CD40L decreases apoptosis in lpr thymocytes

It was not clear from the above findings whether the increased lymphoid cellularity with anti-CD40L treatment resulted from increased proliferation and/or decreased apoptosis. This was examined, using propidium iodide to measure cell cycling and the TUNEL assay to quantitate apoptosis. Cell cycle analysis by propidium iodide revealed only low levels of cell cycling in the thymus, and LN and was no different with anti-CD40L or hamster IgG. Similarly, in vitro culture of normal or lpr LN cells with anti-CD3 demonstrated no augmented proliferation with the addition of anti-CD40L, either soluble or immobilized (data not shown). In contrast, the level of apoptosis in the thymus, as revealed by the TUNEL assay, was considerably lower in the anti-CD40L treatment group. Figure 7A shows the percentage of TCR-αβ+ thymocytes in each subset that bears degraded DNA (numbers in parentheses). Anti-CD40L treatment resulted in diminished levels of degraded DNA among TCR-αβ+ thymocytes in all four subsets defined by CD4 and CD8. This was frequently at least twofold less than observed in control mice. These differences in thymocyte apoptosis were not detectable in the LN, perhaps due to the intrinsically low levels of apoptosis at this site (Fig. 7B). This would suggest that the pronounced increased cellularity of the LN with anti-CD40L might have resulted from increased thymic output of precursors of TCR-αβ+ CD4+CD8- cells rather than in situ expansion of these cells in LN.

To further examine whether the delayed thymocyte apoptosis resulting from anti-CD40L was an in vivo artifact, an analogous study was performed on lpr thymocytes in vitro. Thymocyte suspensions were placed in serum-free medium, and anti-CD40L or hamster IgG was added, followed by cross-linking. Anti-CD3 was then added, and apoptosis was quantitated by TUNEL assay at 3.5
or 5.0 h. Figure 8 shows that, compared with the hamster IgG control, thymocytes pretreated with anti-CD40L before CD3 ligation manifested strikingly less down-regulation of TCR as well as decreased apoptosis, which was present primarily in the TCRlow subset. Table II shows that these findings were highly consistent in three experiments and statistically significant (p < 0.016).

MRL-lpr mice have decreased expression of CD40L

Although thymocyte enlargement with anti-CD40L treatment in vivo has been observed in other strains of normal mice (39), LN expansion has not been reported. The striking lymphoid hyperplasia seen with anti-CD40L treatment of MRL-lpr mice might reflect an increased expression of CD40L by lpr T cells. This was not the case. In both MRL-lpr and MRL +/- mice, unstimulated thymocytes revealed similarly low levels of CD40L expression (Fig. 9A). Following activation with PMA plus ionomycin for 4 h, there was a substantially increased expression of CD40L on MRL +/- thymocytes that was more pronounced on the CD4 CD8 subset compared with the CD4+ CD8+ or other subsets, in agreement with previous findings (39). In contrast, the activated CD4+ CD8+ thymocytes of MRL-lpr mice expressed considerably lower levels of CD40L, as did the CD4+ CD8+ subset (Fig. 9A). A similar difference of CD40L expression was observed in the same subsets of LN cells between MRL +/- and MRL-lpr mice (Fig. 9B). Of particular note is that the TCR-αβ+ CD4+ CD8+ LN cells expressed low to negligible levels of CD40L, either unstimulated or following activation with PMA plus ionomycin. This creates a seeming paradox in that CD40L is not expressed by the lpr LN cell type that accumulates to the greatest degree with anti-CD40L treatment. The explanation for this disparity may lie in the realization that the lineage of TCR-αβ+ CD4+ CD8+ LN cells in lpr mice derives from mature T cells, primarily of the CD8+ subset (40, 41).

Discussion

The current findings show that, in marked contrast to CD40L-deficient/lpr mice, treatment of MRL-lpr mice with anti-CD40L Ab manifested accelerated adenopathy, glomerulonephritis, and death. Although IgG autoantibody production persisted to various degrees with both methods of CD40 signal blocking, the CD40L-deficient/lpr mice developed less disease and little difference in adenopathy compared with standard MRL-lpr mice (32). The collective observations from both systems suggest that in CD40L-deficient/lpr mice the resulting phenotype is due to the actual disruption of the CD40/CD40L axis, whereas administration of anti-CD40L confers an active signal. Our data suggest that this CD40L signal manifests as a partial inhibition of T cell apoptosis beyond that which results from the absence of Fas expression in lpr mice.
The notion that CD40L itself may act as a direct signaling molecule, beyond its capacity to engage CD40, has received only modest attention. Cayabyab et al. (31) reported that CD40 \(^{1}\) P815 cells could costimulate in vitro with CD3 to promote proliferation of CD4 \(^{1}\) cells. Using an in vivo model, administration of soluble CD40-Fc \(^{2}\) to CD40-deficient mice restored germinal center formation, although it did not induce Ig class switch (30). This was interpreted as the ability of soluble CD40-Fc \(^{2}\) to activate T cells.

**FIGURE 7.** Decreased level of thymocyte apoptosis with anti-CD40L treatment. Following 2 wk of twice weekly treatment with hamster IgG (upper row) or anti-CD40L (lower row), thymocytes (A) or LN cells (B) were incubated in serum-free medium at 37°C for 4 h. Cells were then surface stained for expression of CD4, CD8, and TCR-\(\alpha\beta\) followed by staining for degraded DNA using the TUNEL assay. Negative control staining was on the thymocytes from hamster IgG-treated mice and were surface stained with hamster IgG-FITC, and TUNEL staining was performed with all steps except in the absence of biotin-dUTP. Positive TUNEL control staining was on thymocytes from mice that received 2 mg of dexamethasone 18 h previously. Number inserts without parentheses indicate the proportion of total cells expressing TCR-\(\alpha\beta\) with or without degraded DNA. Number inserts within parentheses indicate the percentage of TCR-\(\alpha\beta\) cells containing nicked DNA within each subset defined by CD4 and CD8.

**FIGURE 8.** Anti-CD40L cross-linking inhibits in vitro CD3-induced apoptosis of thymocytes. MRL-\(lpr\) thymocytes were placed in serum-free medium with either control hamster IgG (HlgG) or anti-CD40L followed by goat-anti-hamster IgG and then anti-CD3. At the times indicated, cells were stained for expression of CD4, CD8, TCR-\(\alpha\beta\), fixed, and then stained by the TUNEL assay. Numbers in the CD4 vs CD8 staining indicate the percentage of total cells in each subset. Numbers in the TUNEL histograms indicate the total percentage of cells bearing degraded DNA. Note the lack of TCR down-modulation in the presence of anti-CD40L as well as decreased apoptosis, which was statistically significant over the course of three similar experiments (\(p = 0.016\), see Table II).
directly through CD40L to promote germinal center formation. A further study showed that CD40L-deficient mice have a defect in T cell priming that could not be attributed to defective APC function, since CD40L−/− APC could strongly stimulate CD40+ T cells (27). Although these three reports did not examine apoptosis of T cells, they are consistent with the concept of direct signal transduction via CD40L.

A further study reported that anti-CD40L treatment of mice did prevent the deletion of thymocytes bearing self-reactive TCR-Vβ (39). However, this was also observed in CD40L-deficient mice, and both types of mice manifested diminished thymic expression of B7-2. It was consequently viewed that diminished signaling via B7-2 was in part responsible for the decreased apoptosis. While this may be accurate, the findings are also consistent with a model in which direct signaling through CD40L may also inhibit thymocyte apoptosis. Our observation that in vitro treatment of thymocytes with anti-CD40L prevented CD3-induced TCR down-modulation as well as apoptosis suggests that CD40L signaling might have the effect of decreasing the intensity of TCR signaling on thymocytes. Ligation of CD40L also has been shown to up-regulate expression of cell adhesion molecules, such as ICAM and CD44H (42, 43). Lymphocytes can be protected from apoptosis when they are in contact with various stromal cells, such as fibroblasts (44, 45). Conceivably, part of the rescue from apoptosis by

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**Table II. Decreased apoptosis in vitro of lpr thymocytes following CD40L ligation**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Stimulation</th>
<th>% Apoptotic Cells (TUNEL)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>3.5 h</td>
</tr>
<tr>
<td>1</td>
<td>HIgG + Anti-CD3</td>
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</tr>
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<td></td>
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<td>2</td>
<td>HIgG + Anti-CD3</td>
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<tr>
<td></td>
<td>Anti-CD40L + Anti-CD3</td>
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</tr>
<tr>
<td>3</td>
<td>HIgG + Anti-CD3</td>
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<tr>
<td></td>
<td>Anti-CD40L + Anti-CD3</td>
<td>7.9</td>
</tr>
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</table>

MRL-lpr thymocytes from 8-wk-old female mice were placed in serum-free medium at 37°C and immediately stimulated with either hamster IgG (HIgG) or anti-CD40L, combined with anti-CD3, and then cross-linked with goat anti-hamster IgG at saturating levels. At the time points indicated, samples were taken and surface was stained for CD4, CD8, TCR-αβ, and then stained by the TUNEL assay. Numbers indicate the percentage of total thymocytes bearing degraded DNA. A paired t test of the three experiments yielded a p value of 0.016.

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**Figure 9.** Decreased expression of CD40L by lpr T cells. Thymocytes (A) or LN cells (B) from MRL-++ (upper row) or MRL-lpr (lower row) mice were analyzed either unstimulated or after stimulation for 4 h with PMA plus ionomycin. Cells were then stained for expression of CD4, CD8, TCR-αβ, and CD40L. Shown is the expression of TCR-αβ vs CD40L on the subsets indicated. Number inserts indicate the percentage of positive cells in each quadrant. Only a small proportion of CD4+8− cells of either strain expressed CD40L. However, the CD4+8− subset of MRL-++ T cells manifested significant up-regulation of CD40L within 4 h of stimulation, and this was considerably diminished in the same subset of MRL-lpr mice. As can be inferred from the staining of lpr total LN (B), the major subset of TCR-αβintermediate CD4+8− cells was largely devoid of CD40L expression even after mitogen activation.
CD40L stimulation may result from secondary promotion of T cell adhesion to stromal components.

Anti-CD40L treatment blocks progression of a variety of T cell-mediated autoimmune diseases, including collagen-induced arthritis (22), experimental allergic encephalomyelitis (23), chronic graft-vs-host disease (24), as well as other murine models of lupus such as (SWR × NZB)F1 (SNF1) or (NZB × NZW)F1 mice (25, 26). Anti-CD40L administration during chronic graft-vs-host disease blocked production of IgG anti-chromatin and rheumatoid factor (24). In SNF1 mice, as few as three injections of anti-CD40L given to pre-nephritic mice at 3 mo of age markedly reduced the incidence of nephritis for as long as 12 mo. In addition, autoantibody production was inhibited by this anti-CD40L regimen. This stands in marked contrast to the persistence of some IgG autoantibodies in both CD40L-deficient/lpr mice, and the lpr mice given anti-CD40L in this study. In the case of CD40L-deficient/lpr mice, IgG autoantibodies to small nuclear ribonucleoproteins (snRNPs) were persistent whereas development of anti-DNA Abs and rheumatoid factor was absent (32). With prolonged anti-CD40L treatment of MRL-lpr mice, we also observed moderate decreases in serum IgG1 autoantibodies to ssDNA, Sm Ag, but not chromatin, particularly at later times.

Concurrently, the increased output become profoundly manifest. Along the same line of reasoning, only in the absence of Fas would this increased T cell apoptosis in the periphery occur. Such an increased export of thymocytes might not be observed might be that ligation of CD40L on thymocytes inhibits CD40 molecular activation, as others have reported (39). Whereas CD40L stimulation may result from secondary promotion of T cell adhesion to stromal components, the model might also serve to explain two paradoxes. The first is that, although anti-CD40L diminished apoptosis in the thymus, peripheral lymphadenopathy was not observed. This stands in marked contrast to the persistence of some IgG autoantibodies in the lpr/lpr model of murine systemic lupus erythematosus. In J. Exp. Med. 175:1441.


The role of B cells in lpr/lpr-induced autoimmunity. J. Exp. Med. 180:1295.

References


