CD40 Ligand-Deficient T Cells from X-Linked Hyper-IgM Syndrome Carriers Have Intrinsic Priming Capability

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*J Immunol* 2002; 168:1473-1478; doi: 10.4049/jimmunol.168.3.1473
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Deficiency in CD40 ligand (CD40L) expression is associated with impaired T cell immunity in mouse models and in humans. Previous studies have indicated that this is due to the failure of induction of extrinsic costimulatory molecules. However, other studies have suggested that CD40L is an intrinsic costimulatory molecule. The X-linked hyper-IgM syndrome (XHIM) is a primary immunodeficiency caused by mutations in CD40L, resulting in impaired Ab production and T cell immunity. CD4+ T cells from female carriers of XHIM express a variable degree of normal CD40L based on random X chromosome inactivation. We have examined T cells from XHIM carriers to investigate whether CD40L supports T cell function by acting as an intrinsic costimulator or by induction of other costimulatory molecules by examining coexpression of CD40L and markers of T lymphocyte priming. These carriers provide a unique model for comparison of CD40L-expressing and nonexpressing lymphocytes in that all factors, including immunological experience, are equivalent between the two populations. Our results show that compared with CD40L-deficient T cells, T cells that express CD40L normally have a minimal advantage in becoming primed, as defined by CD45 RO isoform expression and production of IFN-γ and TNF-α. Conversely, CD40L-deficient T lymphocytes clearly were capable of becoming primed as defined by the same parameters. These findings imply that the intrinsic costimulatory activity of CD40L is not required for attaining primed status, and that CD40L primarily supports T cell function by inducing extrinsic factors that can be shared by CD40L-deficient cells. The Journal of Immunology, 2002, 168: 1473–1478.

A ligand that plays a critical role in the adaptive immune response is the CD40 ligand (CD40L, CD154). The requirement for CD40L both in Ab-mediated and in T cell-mediated immunity is evident in the X-linked hyper-IgM syndrome (XHIM), a primary immune deficiency caused by mutations in CD40L, which is characterized by recurrent bacterial as well as T cell opportunistic infections (1). XHIM patients demonstrate a profound deficiency in Ab production due to the failure of CD40L-induced B cell maturation. However, unlike other primary humoral, XHIM patients display a high incidence of T cell opportunistic infections, particularly Pneumocystis carinii pneumonia and Cryptosporidium enteritis. These opportunistic infections provide clear in vivo evidence that CD40L is an essential mediator of T cell immunity.

The profound defect in T cell immunity among XHIM patients has been evident in vitro studies. T cells from XHIM patients respond normally to mitogenic stimulation (1) but are markedly impaired in Ag priming as measured by CD45RO expression, by TNF-α and IFN-γ production, and by the ability to induce IL-12 synthesis (2). In contrast to these findings, one recent study has demonstrated that T cells from XHIM patients show no difference in the ability to make TNF-α, IFN-γ, IL-2, and IL-4 when compared with T cells from age-matched controls (3).

The critical nature of CD40L/CD40 signaling in T cell-mediated immunity also has been corroborated by studies using CD40L-deficient mice, as well as strategies to block CD40L/CD40 interactions. CD40L-deficient mice demonstrate defects in recall response to T cell-dependent Ags (4), in graft-vs-host disease (5), and in inducing experimental allergic encephalomyelitis (6). In addition, blockade of CD40L/CD40 prevents the usual recovery of immune-reconstituted SCID mice from Pneumocystis infection (7). Furthermore, Ab blockade of CD40L can impair T cell mediated inflammation, including graft-vs-host disease (8), rejection of solid organ transplants (9, 10), collagen-induced arthritis (11), and a murine model of lupus nephritis (12).

The induction of costimulatory molecules on CD40-bearing cells is one mechanism by which CD40L/CD40 interactions support T cell function. Ligation of CD40 by CD40L enhances expression of B7.1 and B7.2 molecules on B cells (13), dendritic cells (14), and monocytes (15). Subsequent costimulation of T cells by ligation of CD28 by B7.1 and B7.2 is required for effector T cell differentiation (16). Indeed, the ability to induce experimental allergic encephalomyelitis in CD40L-deficient mice can be restored by overexpression of B7 on APC (6). Furthermore, the capacity of B cells from CD40-deficient mice to induce tolerance to alloantigen can be abolished if the cells are stimulated to express B7 molecules by LPS (17). In addition to B7 molecules, CD40

Received for publication July 6, 2001. Accepted for publication November 19, 2001.

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1 This research was supported by U.S. Public Health Grant 1R29AI40534 (to R.L.F.), by a grant from the Charles Hood Foundation (to R.L.F.), by a Mentored Clinical Scientist Development Award (1K08AB01554-02) from the National Institute of Allergy and Infectious Disease, National Institutes of Health (to F.M.L.), and by a Research Grant from the American Cancer Society, Illinois Division (to P.R.S.).

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3 Abbreviations used in this paper: CD40L, CD40 ligand; XHIM, X-linked hyper-IgM syndrome.
ligation also stimulates production of a variety of cytokines, such as dendritic cell production of IL-12 (18). In this model, the costimulatory molecules and cytokines produced by CD40-bearing cells are extrinsic factors that can be shared by CD40L-deficient T cells in the same microenvironment (Fig. 1A).

In addition to the induction of costimulatory molecules by CD40L, Brenner et al. (19) have provided evidence that CD40L itself might provide an intrinsic costimulatory signal to CD40L-expressing T cells. In their studies, mAb stimulation of CD40L leads to Rac1-dependent activation of Jun N-terminal kinase and p38 MAP kinase. CD40L stimulation also results in tyrosine phosphorylation of a variety of intracellular proteins, including PLCγ, with subsequent activation of protein kinase C (20). Furthermore, the same group has determined that mAb stimulation of CD40L activates a neutral sphingomyelinase, leading to consumption of the same group has determined that mAb stimulation of CD40L with subsequent activation of protein kinase C (20). Furthermore, the same group has determined that mAb stimulation of CD40L activates a neutral sphingomyelinase, leading to consumption of sphingomyelin and generation of ceramide (21), although the significance of this pathway in T cell activation is unknown. Elegant studies by van Essen, Poudrier, Gray, and colleagues (22, 23) have provided further evidence of the role of intrinsic signaling by CD40L in T cell effector function. Stimulation of CD40L by soluble CD40 in a CD40−/− mouse model restores initiation of germinal center formation (22) and enables optimal production of IL-4 by T cells (23). In this intrinsic model of CD40L-derived T cell help (Fig. 1B), any CD40L-deficient T cell would derive no benefit from these means of costimulation and consequently would be at a disadvantage in becoming Ag primed.

Although XHIM patients demonstrate profound defects in B cell- and T cell-mediated immunity, the female carriers of XHIM show little if any immune deficiency. Because CD40L deficiency is not lethal to developing T cells, the random inactivation of the X chromosome in female carriers leads to a mixed population of T cells bearing either normal or defective CD40L (24). We have tested female carriers of XHIM to ascertain whether the failure of T lymphocytes to express functional CD40L conveys an intrinsic inability to become primed, as measured by surface phenotype and the ability to secrete cytokines. Our model requires maximal stimulation of T cells, using phorbol ester and calcium ionophore, to induce CD40L expression on all cells capable of expressing it. Although such conditions of stimulation allow for full expression of CD40L, they induce expression of cytokines such as TNF-α and IFN-γ only on T cells that have been primed by previous Ag stimulation (25). Therefore, coexpression of CD40L and cytokine reflects the contribution that CD40L makes to T cell priming.

Because CD40L-bearing and -deficient T cells in XHIM carriers have matured under identical conditions, the XHIM carriers provide a model for determining the relative advantage conveyed by CD40L in which factors beyond age, most notably immunological experience, are optimally controlled. This avoids the inaccuracies inherent in attempts at matching subjects with immunologically “normal” controls. Indeed, studies of the role of CD40L in T cell priming using patients and matched controls have generated sharply contrasting results (2, 3). The study by Jain et al. (2) indicates that CD40L-deficient T cells from XHIM patients are severely impaired in the ability to make cytokines compared with controls not matched by age. Using age-matched controls, Uronen et al. (3) have demonstrated that T cells from XHIM are equal and in some cases superior to control T cells in cytokine secretion. By examining XHIM carriers, our study addresses this question with a novel and optimal internal control.

Our results indicate that CD40L-expressing lymphocytes have only a minimal advantage over CD40L-deficient T lymphocytes in attaining a primed phenotype as defined by CD45RO expression and the ability to produce TNF-α and IFN-γ. There was no distinction in the ability to make IL-2. These data indicate that CD40L deficiency does not cause an intrinsic T cell priming defect. They also argue that any intracellular signaling by CD40L is not a requirement for T cell priming. However, these results stand in contrast with studies showing no advantage for CD40L-bearing T cells in cytokine production (3). As such, they provide insight into the mechanisms by which CD40L supports T cell immunity.

### Materials and Methods

#### XHIM carriers and controls

Five carriers of XHIM from different families were studied. The diagnosis of XHIM was made in a male relative of each by clinical criteria and/or by CD40L sequencing. Immunologically normal volunteer blood donors served as controls. Informed consent was obtained from all subjects under a protocol approved by the Human Investigation Committee of Yale University.

#### Lymphocyte preparation

PBMC were isolated from whole blood of XHIM carriers and controls by Ficoll density gradient centrifugation, and they were enriched for CD4+ cells by negative selection using affinity chromatography columns (Isocell; Pierce, Rockford, IL), resulting in >97% purity of CD4+ cells as determined by flow cytometry.

#### Flow cytometry

CD4+ T cells were stimulated with 20 ng/ml PMA and 1.5 μM ionomycin for 6 h. They were assayed for cell surface expression of CD40L and CD45RA or CD45RO isomers by flow cytometry on a FACScan (BD Biosciences, San Jose, CA) as described (26) using a PE-conjugated CD40L mAb and FITC-conjugated CD45 mAbs (BD PharMingen, San Diego, CA). Appropriate isotype control mAbs were used for background staining. The total percentage of CD40L+ cells was adjusted by multiplying the percentage of CD40L+ by the percentage of CD4+ purity.

#### Intracellular cytokine staining

FITC-conjugated IL-2, TNF-α, and IFN-γ mAbs (BD PharMingen) were used for two-color staining. At 2 h before the end of the 6-h stimulation,
monensin was added at a 2 μM final concentration. After staining for cell surface CD40L or isotype control, the cells were fixed by overnight incubation at 4°C in 2% paraformaldehyde in PBS. The cells then were washed twice with and resuspended in 100 μL of permeabilization buffer (0.1% saponin and 1% FCS, w/v, in PBS) followed by a 30-min incubation with FITC-conjugated mAbs against IL-2, TNF-α, IFN-γ, or isotype controls. They then were washed in an excess volume of PBS and analyzed by flow cytometry.

**Results**

**XHIM patient characteristics**

The diagnosis of XHIM was made by clinical criteria and by the absence of detectable cell surface CD40L by flow cytometry in a male child of carriers 1, 2, 3, and 5, and in a brother of carrier 4. The brother of carrier 4 has been found to have a splice donor site mutation, leading to exon 3 deletion of CD40L (27). His sister’s carrier status was confirmed by RT-PCR, which demonstrated both the normal and the shortened form of CD40L that corresponds to her brother’s exon 3 deletion (data not shown). The clinical features of each XHIM patient indicate that each family’s mutation resulted in impairment of T cell-mediated immunity (Table I). Four of the five XHIM patients presented with Pneumocystis pneumonia. The XHIM carriers in our study had no clinical evidence of immune deficiency.

**XHIM carriers have partial expression of CD40L**

CD4+ T cells from XHIM carriers were stimulated with a dose of PMA (20 ng/ml) and ionomycin (1.5 μM) that induces CD40L expression on 95–100% of CD4+ T cells from an immunologically normal control (Fig. 2). Based on this degree of CD40L expression after PMA and ionomycin stimulation, we predicted that CD40L should be expressed on virtually all CD4+ cells in which the activated X chromosome contained an intact CD40L gene. Expression of CD40L among carriers ranged from 37 to 69% of CD4+ lymphocytes (Fig. 2), reflecting the random degree of inactivation of X chromosomes expressing either normal or mutated CD40L. The significant proportion of CD4+ lymphocytes that failed to express CD40L indicates that the absence of functional CD40L does not impair T cell maturation and release into the peripheral circulation.

**CD40L-expressing CD4+ T cells have a partial advantage in CD45RO expression**

To determine whether functional CD40L conveys an advantage in T cell Ag priming, we assayed the levels of coexpression of CD40L and CD45RO, a marker of Ag-primed lymphocytes (Fig. 3A and Table II). CD4+ T cells were isolated from XHIM carriers, stimulated with PMA and ionomycin, and analyzed by two-color flow cytometry for coexpression of CD40L and CD45RO. Analysis of the level of coexpression of CD40L and CD45RO revealed that among the CD45RO population, a marginal majority expressed normal CD40L (Table II), dictating that nearly one-half of the primed CD45RO-positive T cell population in XHIM carriers were CD40L-nonexpressing T cells. The degree of advantage for CD40L-expressing cells to become primed was calculated as the ratio of the percentage of CD40L-positive among all primed (CD45RO-positive) T cells to the percentage of CD40L-positive cells in the whole CD4+ population. The range of this ratio was between 1 and 2 in all of the carriers, indicating a marginal advantage for CD40L-expressing T cells in attaining a primed phenotype. However, CD40L+ T cells from carriers with a higher

![Figure 2](http://www.jimmunol.org)
level of normal CD40L expression (e.g., carriers 3, 4, and 5) showed a relatively smaller advantage in attaining CD45RO expression than those from carriers with lower levels of normal CD40L expression (e.g., carriers 1 and 2). Nevertheless, these data demonstrated that T cells that failed to express CD40L were in fact able to become primed in a magnitude nearly that of CD40L-expressing T cells.

**CD40L-expressing CD4⁺ T cells constitute a minority of the naive T cell population**

We further sought to determine whether the advantage that CD40L conveyed for becoming primed correlated with a lower likelihood of CD40L-positive lymphocytes to remain naive, as assessed by expression of the CD45RA isoform of CD45. By an analysis similar to that used for CD45RO coexpression, it was evident that CD40L-bearing T cells were at a relative disadvantage for remaining CD45RA positive (Fig. 3B and Table II). However, this relative disadvantage was minor, with a ratio in all carriers of just under one.

**CD40L-expressing cells have a minor advantage in cytokine production**

We sought to test the primed status of CD40L-positive and -negative T cells functionally by using intracellular cytokine staining to assess their ability to make IL-2, as well as TNF-α and IFN-γ, which reflects prior Ag priming, Th1 differentiation, and commitment to memory cell phenotype (25, 28). CD40L-expressing cells had little or no advantage over CD40L-negative cells in three of four carriers tested (Fig. 4 and Table III). Only in carrier 1, whose CD40L expression was the lowest, did CD40L-positive T cells demonstrate a clear advantage in production of TNF-α and IFN-γ and a smaller advantage in IL-2 production. However, in the other carriers, CD40L-deficient T cells showed little if any impairment in the ability to produce cytokines that characterize primed Th1 lymphocytes. Although the differences were small, T cells from carriers with higher levels of normal CD40L expression (e.g., carriers 3 and 4) had less of an advantage in cytokine production than those from carriers with lower levels of normal CD40L expression (e.g., carriers 1 and 2).

**Discussion**

Numerous studies, both in human patients with XHIM and in mouse models of CD40L deficiency, have demonstrated the importance of CD40L for T cell function. In these models, CD40L-mediated signaling is completely absent. However, the lack of evidence of immune deficiency in the maternal carriers of XHIM indicates that partial expression of CD40L among CD4⁺ T cells is adequate for protective immunity (24). It is possible that the CD40L-expressing population provides all of the effective T cell immunity in XHIM carriers, with no help from the CD40L-deficient fraction. This is supported by the observation that XHIM carriers with extreme lyonization of the X chromosome, resulting in only 5% of T cells expressing functional CD40L, can present

### Table II. Expression of CD40L among CD45RO⁺ and CD45RA⁺ T cells

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Total CD4⁺</th>
<th>Total CD45RO⁺</th>
<th>Total CD45RA⁺</th>
<th>Relative advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CD45RO⁺/CD4⁺</td>
</tr>
<tr>
<td>1</td>
<td>35.8</td>
<td>64.6</td>
<td>27.3</td>
<td>1.8</td>
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<td>2</td>
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<tr>
<td>5</td>
<td>67.3</td>
<td>79.7</td>
<td>60.1</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* Purified CD4⁺ T cells from XHIM carriers were analyzed by flow cytometry for coexpression of CD40L and either CD45RO or CD45RA 6 h after stimulation with 20 ng/ml PMA and 1.5 μM ionomycin, a stimulus sufficient to induce CD40L expression in ≥95% of CD4⁺ T cells from immunologically normal controls.

* The relative advantage for a CD40L⁺ cell to attain the primed CD45RO phenotype was determined by calculating the ratio of the percentage of CD40L⁺ cells among all CD45RO⁺ cells to the percentage of CD40L⁻ cells in the total CD4⁺ population.

* A similar ratio was calculated for CD40L expression among total CD45RA cells to determine the relative advantage for CD40L⁺ cells to have the naive CD45RA phenotype.
with an XHIM phenotype (29). This would be consistent with the
notion that CD40L itself acts as a costimulatory molecule for T
cells, providing help only to cells that express it. An alternate
possibility is that the support that CD40L provides for T cell
immunity is a factor that can be shared by CD40L-deficient T cells,
allowing both populations to contribute to T cell immunity. The
observation that B7 molecules can reverse the T cell dysfunction
in CD40L-deficient mice (6) would be in accord with this model in
which B7 costimulation allows CD40L-deficient T cells to func-
tion as full effectors.

In this study, we have found that CD40L-deficient T cells in
 carriers of XHIM are minimally impaired in comparison with
CD40L-expressing T cells in several parameters of T cell priming.
This stands in contrast with the findings of deficient T cell priming
in complete CD40L-deficient states both in human patients with
XHIM (2) and in CD40L-deficient mice (4). However, our findings
suggest that the presence in carriers of a fraction of T cells ex-
pressing CD40L can allow CD40L-deficient T cells to become
primed T cells. It is not clear whether these primed CD40L-defi-
cient T cells are operative in host defense in a manner that is truly
equal to CD40L-expressing T cells. However, the observation that
they can make TNF-α and IFN-γ with a capacity near that of
CD40L-expressing cells suggests that they are mediating T cell
immunity.

The best reconciliation of these data with the T cell impairments
found in complete CD40L deficiency states would be a model in
which CD40L-deficient T cells can share the support provided by
CD40L-expressing cells. Our observation that the relative advan-
tage for CD40L+ cells decreases in carriers with higher levels of
normal CD40L expression argues for a “bystander effect” that can
be shared in a dose-dependent manner. This model is consistent
with the induction of B7 or other downstream costimulatory mol-
ecules as the major mechanism of CD40L support of T cell immu-
nity, rather than CD40L acting as a costimulatory molecule for
T cells that express it. However, in contrast to the study of Uronen
et al. (3), our study shows a marginal but consistent advantage for
CD40L-expressing T cells in becoming primed. This may reflect
the contribution of intracellular signaling by CD40L in T cell
priming.

These findings also provide important information for the design
of therapeutic strategies for the reconstitution of CD40L expres-
sion by stem cell transplantation or gene therapy. The apparent
ability of CD40L-expressing cells to support the function of
CD40L-deficient cells suggests that the efficiency of correction
need not be higher than the level of CD40L expression found in
XHIM carriers. Because most proposed methods of gene therapy
achieve far less than 100% efficiency in gene correction, our study
provides hope that such strategies may achieve a satisfactory out-
come. Similarly, stem cell transplantation protocols that use non-
myeloablative conditioning regimes that may result in mixed
hematopoietic chimerism would be expected to satisfactorily
reconstitute CD40L-dependent helper T cell functions provided
that the degree of chimerism for CD40L expression is comparable
to that observed in XHIM carriers.

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Total CD40L</th>
<th>Total IFN-γ</th>
<th>Total TNF-α</th>
<th>Total IL-2</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>35.8</td>
<td>59.6</td>
<td>59.7</td>
<td>46.1</td>
</tr>
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<td>40.1</td>
<td>47.0</td>
<td>52.6</td>
<td>49.6</td>
</tr>
<tr>
<td>3</td>
<td>52.3</td>
<td>49.6</td>
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<td>4</td>
<td>67.3</td>
<td>69.0</td>
<td>72.6</td>
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</table>

<table>
<thead>
<tr>
<th>Relative Advantage</th>
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<tbody>
<tr>
<td>IFN-γ+/CD40L</td>
</tr>
<tr>
<td>1.7</td>
</tr>
<tr>
<td>1.2</td>
</tr>
<tr>
<td>0.9</td>
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<tr>
<td>1.0</td>
</tr>
</tbody>
</table>

a Purified CD40L+ T cells from XHIM carriers were analyzed by flow cytometry for coexpression of CD40L and either IFN-γ,
TNF-α, or IL-2 6 h after stimulation with PMA 20 ng/ml PMA and 1.5 μM ionomycin, a stimulus sufficient to induce CD40L
expression in ≥95% of CD4+ T cells from immunologically normal controls.

The relative advantage for a CD40L+ cell to produce each cytokine was determined by calculating the ratio of the
percentage of CD40L+ cells among all cytokine-producing cells to the percentage of CD40L+ cells in the total CD4+ population.
References


