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Direct Physical Interaction Involving CD40 Ligand on T Cells and CD40 on B Cells Is Required to Propagate MMTV

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Summary

The propagation of mouse mammary tumor virus (MMTV) has been analyzed in mice defective for expression of CD40 ligand (CD40L). Mice with endogenous viral superantigen (SAG) delete T cells with cognate Vβ independent of CD40L expression. Nevertheless, CD40L⁻ mice do not show deletion of cognate T cells after being exposed to infectious MMTV and have greatly diminished viral replication. The response of CD40L⁻ T cells to SAG in vitro is also impaired, but can be reconstituted by adding B cells activated by recombinant CD40L to express costimulatory molecules. Thus, direct CD40L-dependent B cell activation appears to be a critical step in the life cycle of MMTV. The initial step in SAG-dependent T cell activation, and hence the MMTV life cycle, may be mediated by non-B cells, because splenocytes from B cell–deficient SAG-transgenic mice are able to activate cognate T cells.

Introduction

The role of the immune system is to limit the spread of infectious agents by mounting a specific and appropriate response against them. This is an efficient strategy, but it is open to subversion. One example of this is the ability of mouse mammary tumor viruses (MMTV) to stimulate a vigorous T cell response by expressing superantigens (vSAG) encoded in an open reading frame in the 3′ long terminal repeat (LTR) (*orf* or *sag*), which stimulate T cells with certain cognate Vβ gene segments expressed on their T cell receptors (TCR). It is thought that this vigorous T cell response stimulates B cells, which initially integrate the virus, and this in turn leads to viral propagation, B cell migration to the mammary gland, and transmission of the virus in milk.

The various components of this scenario have all been tested in earlier experiments. Thus, MMTV cannot be propagated in neonatally thymectomized (Squartini et al., 1970) or nude mice (Tsubura et al., 1988), which lack a thymus and T cells and therefore do not make a T cell response to the virus. The involvement of the *sag* gene in the process was shown by passaging MMTV through mice made transgenic for the same *sag* (Golovkina et al, 1992) or naturally expressing the same endogenous *Mtv* (Held et al., 1993a). Finally, the role of B cells in the process has been implicated by mice lacking B cells, which cannot transmit the virus to their progeny (Beutner et al., 1994), and footpad injection of virus (Held et al., 1993b, 1994b) or ingestion via the gut (Karapetian et al., 1994) leads to a vigorous T–B interaction that is believed to propagate the virus.

Nevertheless, little is known of the mechanism by which this putative T–B interaction takes place. A likely mechanism, based on studies of other T–B interactions, is that stimulation of T cells leads to expression of CD40 ligand (CD40L) (Armitage et al., 1992; Noelle et al., 1992) on the T cell surface, and this then engages CD40 on the B cell with which the T cell is in contact. In this paper, we use a newly derived strain of mice lacking expression of CD40L (Xu et al., 1994) to demonstrate the involvement of this molecule in the propagation of MMTV infection.

Results

CD40L Knockout Mice as a Model System for Studying Cognate T–B Interaction

To investigate the role of CD40L in cognate T–B interaction, we turned to a newly produced strain of mice that lack CD40L due to homologous recombination in embryonic stem cells and transmission in the germ line (Xu et al., 1994). This system is particularly favorable, since the gene for CD40L is on the X chromosome, so heterozygous males are defective in CD40L expression, while heterozygous females are normal. In order for us to use such mice to study MMTV propagation, we had to first document that induction of deletion or functional tolerance occurred normally in CD40L knockout mice.

CD40L Knockout Mice Show Normal Deletion Caused by MMTV (C3H) SAG

CD40L⁻ female mice on a C57BL/6J (B6) genetic background were bred to C3H/HeN, H-2^k mice, which were themselves transgenic for a complete hybrid provirus (HYB–PRO) (Golovkina et al., 1994). The *gag* and *pol* genes in HYB–PRO are derived from Mtv1, while the *env* and *sag* genes originated from exogenous MMTV C3H virus (Shackleford and Varmus, 1988). MMTV(C3H) SAG causes stimulation and deletion of Vβ14- and Vβ15-bearing T cells (Marrack et al., 1991; Choi et al., 1991). In the F1 hybrid generation, all males were CD40L deficient, expressed H-2^k (I-E$^+$), which is a necessary condition for observing deletion by MMTV SAG (Acha-Orbea et al., 1991), and were either MMTV transgene negative or positive. Females in this generation were also MMTV transgene negative or positive, but all expressed CD40L. Table 1 demonstrates that the absence of CD40L did not prevent deletion of Vβ14-bearing cells. The deletion was Vβ14-specific, since Vβ6-bearing cells were present in equal numbers in all groups. Thus, T cells do not need to express CD40L to be deleted by endogenous SAG.

Table 1. CD40L-Deficient Mice Are Able to Delete SAG-Reactive T Cells

	Percent of CD4 T Cells		
Vβ	Male HYB–PRO⁻	Male HYB–PRO⁺	Female HYB–PRO⁺
6	9.7 (10.8; 8.6)	11.7 ± 0.3	11.3 ± 0.7
14	11.0 (11.3; 10.6)	0.9 ± 0.3	0.6 ± 0.1

PBL from male (CD40L⁻) and female (CD40L⁺) mice expressing MMTV C3H SAG (HYB–PRO⁺) or not (HYB–PRO⁻) were stained with anti-CD4 and anti-Vβ antibodies.
Percent of CD4 cells expressing Vβ6 or Vβ14 (mean ± SEM) are shown for groups of 2–4 animals.
If two animals were used, both values are shown.

CD40L Knockout Mice Are Susceptible to Tolerance to an Endogenous Nominal Antigen

Although the data in Table 1 show that SAG can induce tolerance in CD40L knockout mice, we wanted to know whether other forms of tolerance are impaired. Therefore, we took advantage of the fact that H-2^{bxk} mice are functionally tolerant to the peptide Eα52–68 bound to I-A^{b}. We immunized normal or CD40L knockout H-2^{b} mice as well as H-2^{bxk} mice that were normal or defective in CD40L expression with Eα52–68 in complete Freund's adjuvant (CFA) and measured the proliferative response of their lymph node T cells to Eα52–68. Figure 1 shows that all mice responded according to the presence or absence of the peptide, and not according to whether they expressed CD40L. Although the anti-peptide response in CD40L knockout mice was lower than in normal counterparts, one can easily see the difference in reactivity between H-2^{b} versus H-2^{bxk}. In contrast, reactivity to tuberculin, stimulated by CFA injection, was similar in all animals (Figures 1C, 1D). These data strongly support the idea that tolerance is dependent on antigen and not on CD40L expression.

The fact that CD40L-deficient mice are tolerized by either endogenous superantigen or antigen makes it possible to use deletion of Vβ14-bearing T cells as a readout in experiments in which we analyzed the role of T–B interactions on the propagation of MMTV infection in vivo.

Mice Lacking B Cells Due to Gene Knockout Can Also Delete and Activate T Cells with Cognate Vβ Receptors

B cells have been implicated in the propagation of MMTV in infected animals, yet T cells with cognate Vβ are nevertheless deleted in B cell–deficient mice (Beutner et al., 1994). This implies that there must be non-B cell(s) that can participate in T cell recognition of SAG. To test this, we bred μMT mice, which have a deletion in the transmembrane region of Cμ and thus fail to form B cells (Kitamura et al., 1991) to two MMTV transgenic strains of mice. One, the HYB–PRO strain, deleted centrally and was almost devoid of single-positive Vβ14-bearing T cells in both the thymus and the periphery (deletion of about 90%). In the second, the MMTV–ORF19 strain, the transgene consisted of two MMTV C3H LTRs (Golovkina et al, 1992), so that the only RNA it expressed was SAG. Low levels of expression from this transgene led to limited deletion of CD4⁺ T cells in the periphery (36% ± 1.6%) only. No deletion was observed in the CD4⁺ single-positive thymocytes (−3.6% ± 3.2%). No other forms of tolerance could be detected in the thymus: Vβ14 cells reacted normally to stimulation with anti-Vβ monoclonal antibody (MAb) (Golovkina et al., 1992).

Thus, if the results of the cross of μMT mice to these transgenic mice was deletion of Vβ14-bearing cells, it would mean that cells other that B cells can drive these two distinct types of deletion. Table 2 summarizes the data and shows that B cells are not necessary to observe either the high levels of deletion seen with HYB–PRO or the limited peripheral deletion seen with MMTV–ORF19 in the peripheral tissues.

We also asked whether spleen cells from μMT mice

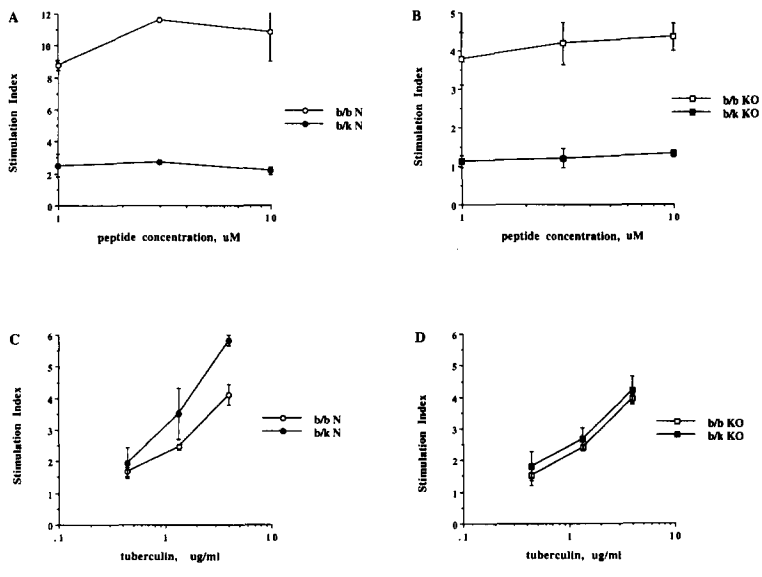

Figure 1. Hyporeactivity to Self-Peptide (Eα 52–68) Is Preserved in CD40L Knockout Mice

Proliferation of regional lymph node cells on day 8 after footpad injection of Eα peptide in CFA and restimulation with peptide (A and B) or tuberculin (C and D) is shown for normal (A, C) or knockout (B, D) mice expressing either H-2^{b} or H-2^{bxk} MHC. Data from individual animals from a representative experiment shown as stimulation index (ratio of experimental to background proliferation for each measurement) ± SEM. KO, knockout; N, normal.

Table 2. Vβ14 T Cells Are Deleted in MMTV Transgenic B Cell–Deficient Mice

Transgene	Percent of Peripheral Vβ14 CD4⁺ T Cells*	
	B Cells Absent	B Cells Present
None	10.7 ± 0.3	9.5 [9.1; 9.9]
HYB–PRO	0.9 ± 0.25 (91.2)	0.7 ± 0.1 (92.4)
MMTV ORF19	6.9 ± 0.3 (35.2)	6.0 [5.7; 6.3] (36.8)

Mean ± SEM. Data from 2–4 animals per group. If two animals were used per group, both values are shown. Deletion (percent), shown in parentheses, was calculated according to the formula: (a − b)/a × 100%, where "a" and "b" was the percent of Vβ14⁺CD4⁺T cells in non-transgenic and transgenic animals, respectively.

could stimulate T cell responses. In Figure 2A, we used a Vβ15-bearing hybridoma to show that it could produce interleukin-2 (IL-2) when stimulated with sufficient numbers of μMT cells, although it is clear that the mice with B cells stimulate these same cells at least 100-fold more

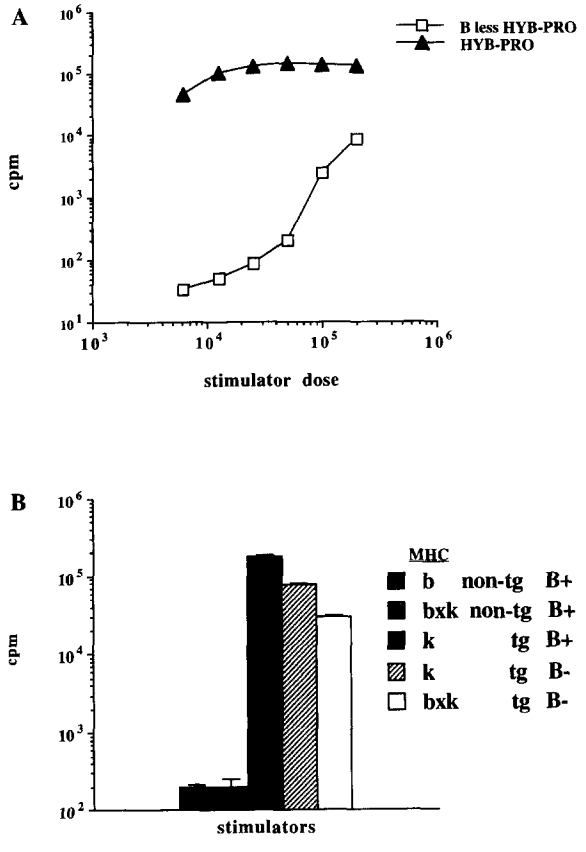

Figure 2. T Cells May Be Activated by Splenocytes from B Cell–Deficient Animals

Spleen cells from B cell–deficient animals, expressing MMTV (C3H) *sag* as transgene were used to stimulate (A) activation of IL-2 production by Vβ15-bearing T cell hybridoma Kox15.8.3 (measured by [³H]thymidine incorporation into the CTLL IL-2–dependent cell line) or (B) proliferation of primary T cells (measured by [³H]thymidine uptake by responding cells) from B cell–sufficient, H-2^{bxk} mouse. tg, HYB–PRO; non-tg, nontransgenic litter mate. B⁺ and B⁻, mice with and without B cells, respectively. Data from representative experiments.

strongly. As shown in Figure 2B, HYB–PRO cells from μMT mice could also stimulate weak but significant responses from MHC-matched naive responder T cells. Thus, non-B cells can drive deletion normally and are potential (though much weaker) stimulators of T cell responses.

CD40L Knockout Mice Fail to Delete Their Vβ14-Bearing T Cells When Exposed to MMTV C3H

To ask whether CD40L was necessary for deletion to occur upon infection with MMTV, two groups of mice (CD40L$^{-/-}$ × B10.BR) F1 were fostered upon infected C3H/HeN or uninfected control mice. It can be seen in Figure 3A that male mice, which lack CD40L, do not delete, while most female mice, which have the ligand, do show deletion (Figure 3B). Moreover, deletion is specific, as it affects Vβ14 but not Vβ6 (see Figure 3, legend). Deletion of Vβ14⁺ T cells in male mice did not occur later in life; at the age of 18–20 weeks, their peripheral blood cells (PBL) contained 8.5% ± 0.7%, while CD40L⁺ mice had only 4.3% ± 0.3% of Vβ14⁺ CD4 cells. Notably, some of the CD40L$^{+/-}$ females (2 of 8 in the experiment shown in Figure 3B) have shown no deletion of cognate T cells. Since CD40L has been shown to be variably expressed in human female carriers of a mutant gene due to random inactivation of X chromosome (Hallenbaugh et al., 1994), the most likely explanation for our observation is variability of the numbers of CD40L⁺ Vβ14⁺ T cells available for interaction with v-SAG at the initial stage of infection. We never observed a failure of deletion in CD40L⁺ males or CD40L$^{+/+}$ females. In subsequent experiments, we crossed MMTV⁻ F1 mice and fostered them on MMTV⁺ milk. This way, we generated males with and without CD40L and with H-2^{bxk} or H-2^{k} backgrounds, which was important because deletion occurs more extensively in H-2^{k} C3H mice (data not shown), suggesting that amount of MHC class II (I-E) expressed is critical for the propagation of infection. Table 3 summarizes staining data obtained from these males. Deletion correlates with expression of CD40L. This shows that inactivation of CD40L protects Vβ14 T cells from deletion, even though deletion itself is stronger in the H-2^{k} homozygous background. Thus, it is clear that MMTV is either not replicating in the absence of CD40L or it does not cause deletion. Since we have already shown that neither CD40L nor B cells are necessary to observe deletion, it seemed more likely that MMTV was not replicating in mice defective for expression of CD40L.

CD40L Is Essential for Propagation of the MMTV Virus In Vivo

To determine whether MMTV was replicating in mice that lack CD40L, we examined viral integration by polymerase chain reaction (PCR) of spleen DNA from male H2^{bxk} mice expressing CD40L or not and exposed to MMTV-infected milk. A unique restriction site on MMTV (C3H) can be revealed by cutting with the enzyme MunI (Beutner et al., 1994), allowing us to discriminate exogenous and endogenous LTRs expressed in splenic DNA. We used a sensitive

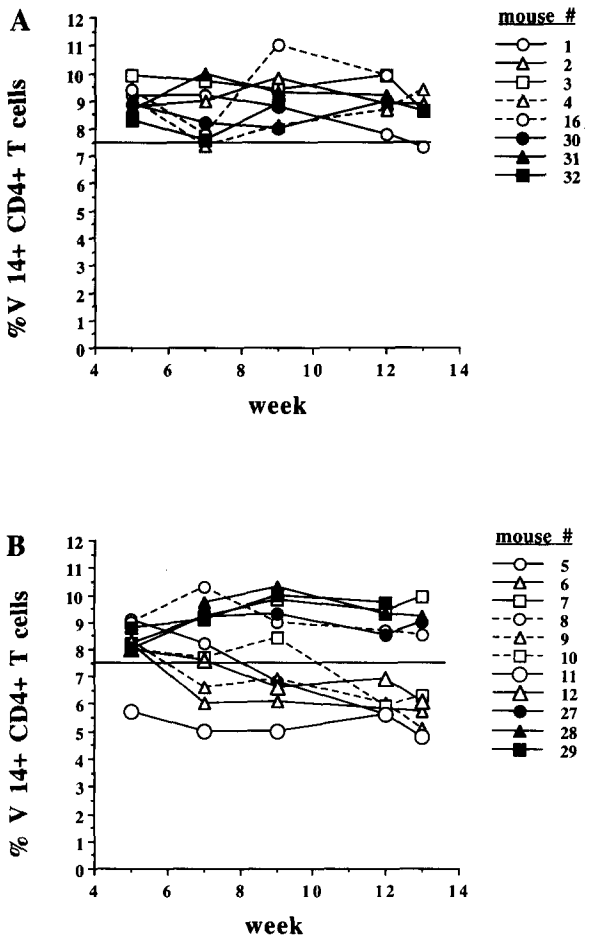

Figure 3. The Failure of T Cells to Express CD40L Prevents Deletion of Cognate T Cells in MMTV-Exposed Animals

CD40L knockout (H-2^b) females were bred to B10.BR/J (H-2^k) normal males. F1 (H-2^{bxk}) mice were foster fed by MMTV$^-$ or MMTV$^+$ C3H/HeN females. Vβ14 plus CD4$^+$ T cells were monitored in PBL of (A), F1 males (CD40L knockout) and (B), F1 females (CD40L$^{+/-}$). MMTV exposed mice are shown in open symbols, mice not exposed to MMTV in closed symbols. Each curve represents an individual animal. Deletion of T cells in CD40L$^{+/-}$ mice is MMTV (C3H) SAG specific: expression of Vβ6 by PBL CD4$^+$ T cells from mice shown in (A) and (B) at the age of 12 weeks was 8.7% ± 0.5% and 8.5% for exposed and nonexposed males and 9.2% ± 0.6% and 7.8% ± 0.5% for exposed and nonexposed females, respectively.

assay of probing with an LTR probe on filters to which the PCR product was transferred either before or after digestion with MunI (Figure 4). Only MMTV-specific fragment (600 bp, top band) is digested by Mun I into smaller fragments, the bigger of which is revealed by hybridization. The nature of intermediate band also amplified in infected animals is unknown. This analysis revealed that substantial amount of MMTV provirus could be found in the spleens of CD40L-sufficient mice, but not in the spleens of uninfected animals, and could be barely detected in CD40L-defective mice (Figure 4, lanes 4 and 8) and only after 3 day exposure of the X-ray film. Consistent with the hybridization data, mice without CD40L, exposed to infectious milk, show no deletion of Vβ14$^+$ CD4$^+$ PBL.

Moreover, MMTV-exposed CD40L$^-$ females do not transmit the virus (or transmit extremely low amounts) to their progeny. When the progeny of such females was analyzed at the age of 9 weeks, the percent of Vβ14$^+$ CD4$^+$ PBL was 8.4% ± 0.9% (n = 7), while the progeny of CD40L$^+$ females had only 5.3% ± 1.5% Vβ14$^+$ CD4$^+$ (n = 5). Control MMTV$^-$ mice contained 9.0% ± 0.7% of Vβ14$^+$ CD4$^+$ PBL (n = 3).

Thus, three pieces of data strongly implicate CD40L in the propagation of MMTV in vivo. First, there is no deletion of Vβ14-bearing T cells in these mice. Second, there is only barely detectable viral integration in splenic DNA. And third, CD40L-deficient mice apparently do not transmit the MMTV to their progeny.

Role for Direct Contact of T Cells with CD40L with B Cells Expressing CD40 in Propagation of MMTV

The data leave one possible caveat with regard to the role of T-dependent B cell activation mediated by CD40L. That is, we have not formally shown that the T cells can respond to SAG without expressing CD40L. To examine this, we took advantage of insect cell membranes containing CD40L (Kehry and Castle, 1994). When T cells from normal or CD40L-defective mice are used as responders in mixed lymphocyte culture, it can be seen that T cells from CD40L knockout mice give a very low response to HYB-PRO B cells compared with T cells from normal animals (Figure 5A). However, 48 hr of culture of HYB-PRO B cells with insect cell membranes expressing CD40L induces strong responses from all T cells, whether they express CD40L or not (Figure 5B), and it is accompanied by strong induction of B7-2 (Figure 5C), which is the relevant costimulator. To prove that the observed activation was due to T cell–independent activation of costimulatory activity, we showed that T cell response could be efficiently blocked by CTLA-4Ig (Linsley et al., 1991) (Figure 5D), a ligand for B7-1, B7-2, and possibly other costimulatory molecules. Thus, T cells without CD40L can respond, provided the B cells are preactivated.

Discussion

In the present experiments, we have shown that CD40L-deficient mice and B cell–deficient mice can drive deletion of Vβ14-bearing T cells, provided the MMTV (C3H) SAG is expressed by an endogenous virus. What CD40L-deficient mice cannot do is allow the propagation of the virus acquired through milk. Thus, the CD40L–CD40 interaction plays a key role in viral life cycle.

Milk laden with virus is ingested, and infects some cell type that bears MHC class II on its surface. Possibilities include M cells, gut epithelial cells (Kaiserlian et al., 1993), Langerhans cells, dendritic cells, macrophages, and B cells. It is a consensus view that SAG-dependent T cell activation is a key event in the MMTV life cycle (Golovkina et al, 1992; Held et al., 1994a). The results with μMT mice rule out the B cell as the exceptional cell for initiating the T cell response (see Figure 2B). A possibility that can not be excluded is that initial activation of T cells may be pro-

Table 3. No Deletion of Vβ14⁺CD4⁺T Cells Can Be Seen in CD40L-Deficient Males Foster-Fed by MMTV-Infected C3H/HeN Females

MHC of foster-fed mice	Experiment number	MMTV[a]	CD40L	Percent of Vβ14⁺ CD4⁺T cells[b]	n
bxk	1	−	+	8.55 (8.1; 9.0)	2
bxk	1	+	+	6.0 ± 0.7	3
bxk	1	+	−	9.0 ± 0.6	4
bxk	2	−	+	8.8 ± 0.4	3
bxk	2	−	−	8.1 ± 0.5	4
bxk	2	+	+	5.4 ± 0.3	3
bxk	2	+	−	8.25 (8.9; 8.6)	2
k	2	−	+	7.55 ± 0.4	4
k	2	−	−	7.3	1
k	2	+	+	4.6 (4.4; 4.8)	2
k	2	+	−	7.55 (7.3; 7.8)	2

[a] Mice were fostered by MMTV⁺ or ⁻ C3H/HeN females.
[b] Percent of Vβ14⁺CD4⁺PBL ± SEM, where applicable. Data is shown for 10-week-old animals. H-2^{bxk} mice were either F1(B6.CD40LKO × B10.BR) or their F2. H-2^{k} MMTV⁻CD40L⁺ mice were age-matched B10.BR.
n, number of animals per group.

vided by cells that express SAG but have not integrated MMTV. For example, gut epithelial cells have been shown to express viral proteins, but no viral DNA (Karapetian et al., 1994). It has also not been disproved that virions contain SAG polypeptides. An important finding that inhibition of reverse transcriptase by 3′-azido-3′-deoxythymidine (AZT) given to mice prior to injection of MMTV particles (Held et al., 1994b) abrogated proliferative reaction of T and B cells in the regional lymph nodes does not exclude the possibility that initial stimulation of CD40L expression by T cells was caused by B cells or another cell type without integration of the viral genome. It does argue, though, that involvement of B cells in the proliferative reaction is rapid (within 24–48 hr after injection) and requires reverse transcription.

In normal mice naturally exposed to MMTV, the initiating cell (B or non-B) stimulates T cells, which in turn express CD40L and activate infected B cells, driving replication of the virus. There may be a few B cells infected, which is insufficient to promote the spread of the virus without amplification by several cycles of T–B interactions, dependent on CD40L–CD40 interactions. This interaction induces B7-2 on the B cell, further expanding the T cell pool reactive to SAG, which in its turn stimulates further the B cell proliferation and MMTV propagation. It has been shown in experiments with footpad injections of MMTV into adult animals that the majority of proliferating B cells express surface immunoglobulin G (IgG), suggesting that preactivated B cells were the primary targets of infection (Held et al., 1994b). We would like to suggest that the IgG expression results from activation of the CD40L–CD40 pathway, which triggers the isotype switch (Hodgkin et al., 1990; Noelle et al., 1990; Gascan et al., 1991; Aruffo et al., 1993). An important difference between adult and newborn animals is in the absence of preactivated B cells in the latter, so that it is hard to draw parallels in the two experimental systems.

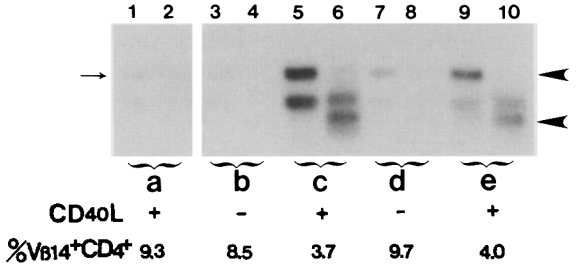

Figure 4. CD40L-Deficient Mice Show Low Integration of MMTV in Splenic DNA

MMTV-specific PCR products were amplified from equal amount of DNA from the spleens of H-2^{kxb} male mice born from CD40L$^{+/-}$ H-2^{kxb} females and normal C3H/HeN males and fostered on MMTV⁻ (a) or MMTV⁺ milk (b–e). Animals b and d were CD40L negative. Amplified fragments were digested with MunI (lanes 2, 4, 6, 8, 10) or mock-treated (lanes 1, 3, 5, 7, 9), gel separated, transferred to nylon membrane, and hybridized to LTR probe. MMTV (C3H)-specific products are indicated by arrowheads. PCR product apparently amplified from an endogenous viral gene and having the same size as uncut exogenous MMTV product (600 bp) is marked by arrow. Percent of Vβ14⁺ CD4⁺ PBL is shown for each mouse at the age of 9 weeks.

Why the activation process ceases, with apoptosis of the responding T cells, is a mystery not yet solved. Suffice it to say that the mechanism of activation by SAG, as opposed to activation by peptide antigen, is probably quite different (Liu et al., 1991; Weber and Cantor, 1994). Notably, deletion of cognate T cells is a slow process, which can be detected in the periphery starting at fourth week postpartum (Marrack et al., 1991). Both CD4 and CD8 cells are being deleted, implicating the participation of the thymus in this process (Ignatowicz et al., 1992). There may be other sites in the organism that control the deletion process, including gut (Webb and Sprent, 1993). From our data and the data by Beutner et al., (1994) with μMT mice, it is obvious that non-B cells may mediate deletion in the periphery as well as in the thymus.

There are further interesting consequences of this model. The deletion is a progressive process, suggesting that it may be of importance to MMTV, rather than being

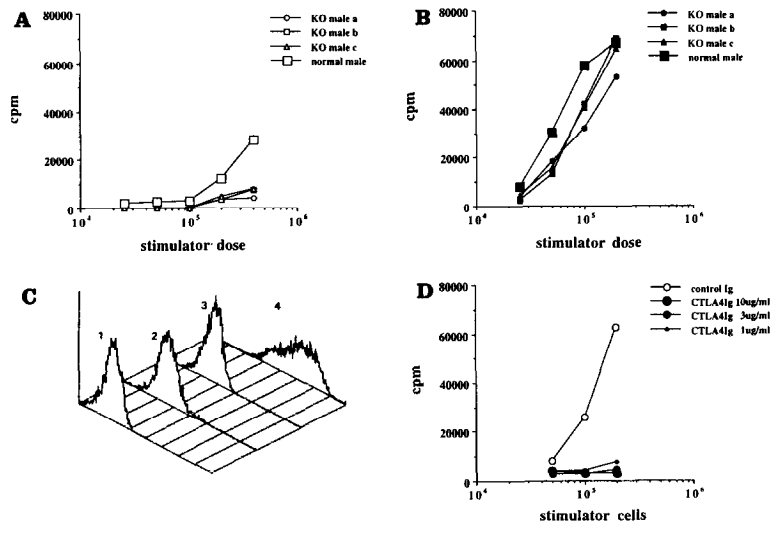

Figure 5. Activation of Costimulatory Molecules on Stimulator Cells Turns Low-Responder CD40L Knockout T Cells into High Responders to HYB–PRO

Comparison of the proliferative responses (measured by [^{3}H]thymidine incorporation) of lymph node cells from normal and CD40L knockout animals to (A) splenic B cells or (B) CD40L-activated B cells from HYB–PRO transgenic mice. Stimulation by splenic B cells and activated B cells from nontransgenic animals treated in the same manner served as a negative control in this experiment and non-SAG-specific [^{3}H]thymidine uptake was subtracted from the data shown.

(C) Expression of B7-2 by B220$^+$ cells from HYB–PRO mice, stained before (1, 2) and after (3, 4) stimulation with insect cell membranes, expressing CD40L. Peaks 1 and 3, control staining; 2 and 4, staining with anti-B7-2 MAb.

(D) CTLA-4Ig (common ligand for B7-1 and B7-2 costimulatory molecules) blocks proliferative response of CD40L knockout lymph node cells against CD40L-activated B cells. Final concentrations of CTLA-4Ig were 10, 3, and 1 μg/ml; final concentrations of control immunoglobulin, 10 μg/ml.

an uncontrolled devastation of cells that have fulfilled their function. Since SAG-reactive T cells are able to activate the costimulatory function of SAG-expressing cells, they may participate in an antiviral immune response. Resting B cells have been shown to be tolerogens rather than stimulators for naive T cells (Eynon and Parker, 1992; Fuchs and Matzinger, 1992). To activate antiviral immunity they might need to express costimulatory molecules, which may be achieved by SAG–TCR plus CD40L–CD40 interaction with cognate T cells. Unlike the activation of T cells by SAG, their deletion does not require the expression of costimulatory molecules (Jones et al., 1993) and, hence, may be easily achieved by SAG presented by various cell types. The virus may use the ability to delete the responding T cells to maintain itself in the host organism until the mammary gland is infected and transmission to the offspring is inevitable.

Finally, which cell is used as a vehicle to bring MMTV to the mammary gland is not clear. It has been shown that infection in adult animal is multidirectional (Held et al., 1994a) and may be propagated by B cells or CD4 and CD8 subsets of T cells (Waanders et al., 1993).

Experimental Procedures

Mice

CD40L-deficient mice were described (Xu et al., 1994). The strain has been continuously bred to C57BL/6J (B6). B10.BR/J were purchased from Jackson Labs (Bar Harbor, Maine). C3H/He/N both MTV$^+$ and MTV$^-$ were from National Institutes of Health (Frederick, Maryland). MMTV transgenic mice (HYB–PRO and MTV ORF19) were a gift from Dr. S. R. Ross (University of Pennsylvania, Philadelphia, Pennsylvania). μMT mice were a gift from Dr. K. Rajewsky (University of Cologne, Cologne, Federal Republic of Germany).

Flow Cytometery

PBL or spleen cells were stained and analyzed as described (Golovkina et al., 1992). MAbs against mouse CD4 and CD8 molecules directly conjugated to R-phycoerythrin or RED613 were from GIBCO BRL (Grand Island, New York). Anti-Vβ-specific MAbs (anti-Vβ6, Kanagawa, 1989; and anti-Vβ14, Liao et al., 1989) conjugated to fluorescein isothiocyanate as well as biotinylated anti-B7-2 (Hathcock et al., 1993) were from PharMingen (San-Diego, California). Biotinylated antibodies were followed by streptavidin–fluorescein isothtiocyanate conjugate (Caltag Laboratories, San Francisco, California).

In Vitro Cell Activation Assays

T cells were activated by cocultivation of 3 × 10^5 lymph node cells with 5 × 10^5 irradiated (2000 rada) or mitomycin c (Boehringer Mannheim GmBH, Mannheim, Federal Republic of Germany) treated splenocytes (30 min at 37°C, 50 μg/ml of mitomycin c). Proliferation of responding cells was quantified by [^{3}H]thymidine incorporation. The reactivity of a T cell hybridoma Kox15–8.3 (Kappler et al., 1988) was tested by cocultivation of 5 × 10^4 hybridoma cells with variable numbers of stimulator cells for 16 hr. Supernatants were tested for IL-2 production by CTLL IL-2-dependent cell line proliferation. All assays were done in duplicate or triplicate cultures in 96-well flat-bottomed plates (Becton-Dickinson, Lincoln Park, New Jersy) in complete Click's EHAA medium with 5% fetal calf serum as described (Golovkina et al., 1992).

B Cell Activation

Spleen cells were depleted of T cells by treatment with MAbs against Thy1.2 (Marshak-Rothstein et al., 1979), CD4 (Dialynas et al., 1983), and CD8 (Ledbetter and Herzenberg, 1979) followed by rabbit complement (Cederlane Laboratories, Limited, Horby, Ontario, Canada) and dead cells removal by density gradient centrifugation. Cells (10^7) were then cultured for 48 hr at 37°C in the presence of a 1% preparation of insect cell membranes (Kehry and Castle, 1994), expressing gp39 (CD40L) protein in Click's EHAA medium supplemented with 5% fetal calf serum. Cells were treated with mitomycin C, extensively washed, and used as stimulators in a T cell activation assay. Cells from nontransgenic C3H/He/N treated exactly the same way were used as negative control and the values of resulting nonspecific [^{3}H]thymidine uptake were subtracted from the data. Samples of activated cells were stained with anti-B220 (Coffman, 1983) conjugated to phycoerythrin (PharMingen) (more than 90% positive) and biotinylated anti-B7-2. Purified CTLA-4Ig chimeric protein (Linsley et al., 1991), used to block the anti-HYB–PRO response was a gift from Dr. P. S. Linsley (Bristol-Myers Squibb Company, Seattle, Washington).

In Vivo T Cell Activation

Mice were injected in the footpads with 30 ng of Eα52–68 peptide in

CFA (DIFCO Laboratories, Detroit, Michigan). Peptide was synthesized by Yale University W. M. Keck Biotechnology Resource Center. Regional lymph nodes were removed at day 8 after immunization. Proliferative responses of 4 × 10^5 lymph node cells per well of 96-well plates were tested in triplicate after stimulation for 48 hr with either antigenic peptide, irrelevant peptide derived from invariant chain (no response was detected in all cases), or tuberculin preparation.

Detection of MMTV Provirus

DNA was extracted from spleens of 9-week-old mice exposed to infected and noninfected milk. PCR conditions and primers were exactly as described (Beutner et al., 1994). Amplified products were cut with MunI (GIBCO BRL) restriction enzyme to distinguish exogenous and endogenous viral LTR and run over 1.2% agarose gel, transferred to nylon membranes (Zeta-Probe, GT, Bio-Rad Labotratories, Richmond, California), and hybridized to ^{32}P-labeled LTR probe (Golovkina et al.,1992), a gift from Dr. T. Golovkina (University of Pennsylvania), according to Southern (1975).

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References

Acha-Orbea, H., Shakhov, A. N., Scarpellino, L., Kolb, E., Muller, V., Vessaz-Shaw, Fuchs, R., Blöchingler, K., Rollini, P., Billote, J., Sarafidou, M., MacDonald, H. R., and Diggelman, H. (1991). Clonal deletion of Vβ14 positive T cells in mammary tumor virus transgenic mice. Nature *350*, 207–211

Armitage, R. J., Fanslow, W. C., Strockbine, L., Sato, T. A., Clifford, K. N., Macduff, B. M., Anderson, D. M., Gimpel, S. D., Davis-Smith, T., Maliszewski, C. R., Clark, E. A., Smith, C. A., Grabstein, K. H., Cosman, D., and Spriggs, M. K. (1992). Molecular and biological characterization of a murine ligand for CD40. Nature *357*, 80–82.

Aruffo, A., Farrington, M., Hollenbaugh, D., Li, X., Milatovich, A., Nonoyama, S., Bajorath, J., Grosmaire, L. S., Stenkamp, R., Neubauer, M., Roberts, R. L., Noelle, R. J., Ledbetter, J. A., Francke, U., and Ochs, H. D. (1993). The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. Cell *72*, 291–300.

Beutner, U., Kraus, E., Kitamura, D., Rajewsky, K., and Huber, B. T. (1994). B cells are essential for murine mammary tumor virus transmission, but not for presentation of endogenous superantigens. J. Exp. Med. *179*, 1457–1466.

Choi, Y., Kappler, J. W., and Marrack, P. (1991). A superantigen encoded in the open reading frame of the 3′ long terminal repeat of the mouse mammary tumor virus. Nature *350*, 203–207.

Coffman, R. L. (1983). Surface antigen expresion and immunoglobulin gene rearrangement during mouse pre-B cell development. Immunol. Rev. *69*, 5–23.

Dialynas, D. P., Wilde, D. B., Marrack, P., Pierres, A., Wall, K. A., Havran, W., Otten, G., Loken, M. R., Pierres, M., Kappler, J., and Fitch, F. W. (1983). Characterization of the murine L3T4a, recognized by monoclonal antibody GK1.5: expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity. Immunol. Rev. *74*, 29–56.

Eynon, E. E., and Parker, D. C. (1992). Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens. J. Exp. Med. *175*, 131–138.

Fuchs, E. J., and Matzinger, P. (1992). B cells turn off virgin but not memory T cells. Science *258*, 1156–1159.

Gascan, H., Gauchat, J. F., Roncarolo, M. G., Yssel, H., Spits, H., and de Vries, J. E. (1991). Human B cell clones can be induced to proliferate and switch to IgE and IgG4 synthesis by IL-4 and a signal provided by activated CD4$^+$ T cell clones. J. Exp. Med. *173*, 747–750.

Golovkina, T. V., Chervonsky, A., Dudley, J. M., and Ross, S. R. (1992). Transgenic mouse mammary tumor virus superantigen expression prevents viral infection. Cell *69*, 637–645.

Golovkina, T. V., Chervonsky, Prescott, J. A., Janeway, C. A., Jr., and Ross S. R. (1994). The mouse mammary tumor virus envelope gene product is required for superantigen presentation to T cells. J. Exp. Med. *179*, 439–446.

Hallenbaugh, D., Wu, L. H., Ochs, H. D., Nanoyama, S., Grosmaire, L. S., Ledbetter, J. A., Noelle, R. J., Hill, H., and Aruffo, A. (1994). The random inactivation of the X chromosome carrying the defective gene responsible for X-linked hyper IgM syndrome (X-HIM) in female carriers of HIGM1. J. Clin. Invest. *94*, 616–622.

Hathcock, K. S., Laszlo, G., Dickler, H. B., Bradshaw, J., Linsley, P., and Hodes, R. J. (1993). Identification of an alternative CTLA-4 ligand costimulatory for T cell activation. Science *262*, 905–907.

Held, W., Waanders, G. A., Shakhov, A. N., Scarpellino, L., Acha-Orbea, H., and MacDonald, H. R. (1993a). Superantigen-induced immune stimulation amplifies mouse mammary tumor virus infection and allows virus transmission. Cell *74*, 529–540.

Held, W., Shakhov, A. N., Izui, S., Waanders, G. A., Scarpellino, L., MacDonald, H. R., and Acha-Orbea, H. (1993b). Superantigen-reactive CD4$^+$ T cells are required to stimulate B cells after infection with mouse mammary tumor virus. J. Exp. Med. *177*, 359–366.

Held, W., Acha-Orbea, H., MacDonald, H. R., and Waanders, G. A. (1994a) Superantigens and retroviral infection: insights from mouse mammary tumor virus. Immunol. Today *15*, 184–190

Held, W., Waanders, G., Acha-Orbea, H., and MacDonald, H. R. (1994b). Reverse transcriptase-dependent and -independent phases of infection with mouse mammary tumor virus: implications for superantigen function. J. Exp. Med. *180*, 2347–2351.

Hodgkin, P. D., Yamashita, L. C., Coffman, R. L., and Kehry, M. R. (1990). Separation of events mediating B cell proliferation and Ig production by using T cell membranes and lymphokines. J. Immunol. *145*, 2025–2034.

Ignatowicz, L., Kappler, J., and Marrack, P. (1992). The effects of chronic infection with a superantigen-producing virus. J. Exp. Med. *175*, 917–923.

Jones, L. A., Izon, D. J., Nieland, J. D., Linsley, P. S., and Kruisbeek, A. M. (1993). CD28–B7 interactions are not required for intrathymic clonal deletion. Int. Immunol. *5*, 503–512.

Kaiserlian, D., Vidal, K., MacDonald, H. R., and Grosjean, I. (1993). Mouse intestinal epithelial cells express the self superantigen Mls1^a. Eur. J. Immunol. *23*, 2717–2720.

Kanagawa, O. 1989. In vivo T cell tumor therapy with monoclonal antibody directed to the Vβ chain of T cell antigen receptor. J. Exp. Med. *170*, 1513–1519.

Kappler, J. W., Staerz, U., White, J., and Marrack, P. C. (1988). Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. Nature *332*, 35–46.

Karapetian, O., Shakhov, A. N., Kraehenbuhl, J.-P., and Acha-Orbea, H. (1994). Retroviral infection of neonetal Peyer's patch lymphocytes: the mouse mammary tumor virus model. J. Exp. Med. *180*, 1511–1516.

Kehry, M. R., and Castle, B. E. (1994). Regulation of CD40 ligand expression and use of recombinant CD40 ligand for studying B cell growth and differentiation. Sem. Immunol. *6*, 287–294.

Kitamura, D., Roes, J., Kühn, and Rajewsky, K. (1991). A B cell–deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene. Nature *350*, 423–426.

Ledbetter, J. A., and Herzenberg, L. A. (1979). Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. *47*, 63–90.

Liao, N., Maltzman, J., and Raulet, D. H. (1989). Positive selection determines T cell receptor Vβ 14 gene usage by CD8 T cells. J. Exp. Med. *170*, 135–143.

Linsley, P. S., Brady, W., Urnes, M., Grosmaire, L. S., Damle, N. K., and Ledbetter, J. A. (1991). CTLA-4 is a second receptor for the B cell activation antigen B7. J. Exp. Med. *174*, 561–564.

Liu, H., Lampe, M. A., Iregui, M. V., and Cantor H. (1991). Conventional antigen and superantigen may be coupled to distinct and cooperative T cell activation pathways. Proc. Natl. Acad. Sci. USA *88*, 8705–8709.

Marrack, P., Kushnir, E., and Kappler, J. (1991). A maternally inherited superantigen encoded by a mammary tumor virus. Nature *349*, 525–526.

Marshak-Rothstein, A., Fink, P., Gridley, T., Raulet, D. H., Bevan, M. J., and Gefter, M. L. (1979). Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. J. Immunol. *122*, 2491–2497.

Noelle, R. J., Daum, J., Barlett, W. C., McCann, J., and Shepherd, D. M. (1990). Cognate interactions between helper T cells and B cells. V. Reconstitution of helper T-cell function using purified plasma membranes from activated Th1 and Th2 helper T cells and lymphokines. J. Immunol. *146*, 1118–1124.

Noelle, R. J., Roy, M., Shepherd, D., Stamenkovic, I., Ledbetter, J. A., and Aruffo, A. (1992). A 39-kDa protein on activated T cells binds CD40 and transduces the signal for cognate activation of B cells. Proc. Natl. Acad. Sci. USA *89*, 6550–6554.

Shackleford, G. M., and Varmus, H. E. (1988). Construction of a clonable, infectious, and tumorigenic mouse mammary tumor virus provirus and a derivative genetic vector. Proc. Natl. Acad. Sci. USA *85*, 9655–9659.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. *98*, 503–517.

Squartini, F., Olivi, M., and Bolis, G. B. (1970). Mouse strain and breeding stimulation as factors influencing the effect of thymectomy on mammary tumorigenesis. Cancer Res. *30*, 2069–2072.

Tsubura, A., Inaba, M, Imai, S., Murakami, A., Oyaizu, N., Yasumizu, R., Ohnishi, Y., Tanaka, H., Morii, S., and Ikehara, S. (1988). Intervention of T-cells in transportation of mouse mammary tumor virus (milk factor) to mammary gland cells in vivo. Cancer Res. *48*, 6555–6559.

Xu, J., Foy, T. M., Laman, J. D., Elliott, E. A., Dunn, J. J., Waldschmidt, T. J., Elsemore, J., Noelle, R. J., and Flavell, R. A. (1994). Mice deficient for the CD40 ligand. Immunity *1*, 423–431.

Waanders, G. A., Shakhov, A. N., Held, W., Karapetian, O., Acha-Orbea, H., and MacDonald, H. R. (1993). Peripheral T cell activation and deletion induced by transfer of lymphocyte subsets expressing endogenous or exogenous mouse mammary tumor virus. J. Exp. Med. *177*, 1359–1366.

Webb, S. R., and Sprent, J. (1993). Factors controlling the reactivity of immature and mature T cells to Mlsa antigens *in vivo*. Immunol. Rev. *131*, 168–188.

Weber, G. F., and Cantor, H. (1994). Phosphatidylinositol synthesis is a proximal event in intracellular signalling coupled to T cell receptor ligation: differential induction by conventional antigen and retroviral superantigen. J. Immunol. *152*, 4433–4443.