Clonal Deletion Versus Clonal Anergy: The Role of the Thymus in Inducing Self Tolerance

FRED RAMSDELL AND B. J. FOWLKES*

During development in the thymus, T cells are rendered tolerant to self antigens. It is now apparent that thymocytes bearing self-reactive T cell receptors can be tolerized by processes that result in physical elimination (clonal deletion) or functional inactivation (clonal anergy). As these mechanisms have important clinical implications for transplantation and autoimmunity, current investigations are focused on understanding the cellular and molecular interactions that generate these forms of tolerance.

THE THYMUS IS RESPONSIBLE FOR GENERATING MATURE functional T cells with a diverse set of T cell receptors (TCRs) for the recognition of foreign antigens (*1, 2*). These antigens are recognized usually as a peptide embedded within major histocompatibility complex (MHC) molecules. Thus, the MHC is a gene cluster that encodes proteins whose function is to bind foreign peptides for presentation to T cells. In the mouse, MHC molecules are grouped according to tissue distribution: those molecules (termed K, D, and L) present on nearly all cells are designated as class I, whereas class II molecules (termed I-A and I-E) are normally present only on thymic epithelium and so-called antigen-presenting cells (usually macrophages, B cells, or dendritic cells). The actual recognition unit of the TCR that binds this MHC antigenic complex is composed of a polyclonally distributed protein heterodimer ($\alpha\beta$ or $\gamma\delta$). Each heterodimer is noncovalently linked to five invariant protein chains that appear to be involved in signal transduction (*3*). Accessory molecules, either CD4 or CD8, can also be an intimate part of this receptor complex. These molecules may serve to enhance the avidity of the TCR for antigen by binding directly to nonpolymorphic portions of MHC molecules (*4*) or may participate in signal transduction (*5*).

The earliest cells to appear in the thymus lack surface CD4 and CD8 as well as TCR and are derived from the fetal liver or adult bone marrow (Fig. 1). These precursor cells rapidly undergo rearrangements of their TCR genes and start to express CD4 and CD8. Those $CD4^+8^+$ cells that successfully rearrange their TCR display receptors on their surface (usually at low levels), thereby constituting a diverse population of cells, each of which bears a unique antigen receptor. This heterogeneity creates the potential to interact with the multiple allelic forms of MHC molecules existing in the different individuals of a species. Within a given individual, however, only some thymocytes will exhibit TCRs that are able to interact with the particular MHC molecules present on the thymic epithelium.

Subsequent to the appearance of $CD4^+8^+$ thymocytes, the $CD4^+8^-$ and $CD4^-8^+$ thymocytes arise that possess high levels of TCR. This differentiation may result from the specific interaction of a particular TCR-bearing thymocyte with MHC molecules, or MHC plus peptide. Alternatively, the appearance of $CD4^+8^-$ and $CD4^-8^+$ cells could occur simply by a stochastic process (*6*). Nevertheless, it appears that a specific interaction between the TCR and MHC molecules, either at the $CD4^+8^+$ or $CD4^+8^-$ or $CD4^-8^+$ stage, rescues the cell from programmed death (termed positive selection). Thus, selective events within the thymus shape the final TCR repertoire, with the result that foreign antigenic peptides are recognized only in association with self-MHC molecules (*7*). The result of this selection is two sets of mature T cells: one set that uses CD4 and recognizes foreign antigen complexed to class II MHC molecules, and a second set that uses CD8 and recognizes antigens complexed with class I MHC molecules.

Another function of the thymus is to impart to the mature immune system the ability to distinguish self from nonself. That is, those T cells capable of responding to self antigens are negatively selected. Experiments that show T cells are tolerant to self antigens only in the context of thymically expressed MHC molecules suggest that negative selection involves recognition of thymic self-MHC molecules (*8*). This implies that tolerance to self can result from a TCR-MHC interaction in the thymus that is similar to the recognition of foreign antigen that activates a mature T cell. Evidence is accumulating that indicates there may be multiple mechanisms for attaining self tolerance and that these mechanisms may operate at various stages of development. Three general mechanisms have been proposed: (i) physical deletion, (ii) functional inactivation, and (iii) suppression of self-reactive clones. Here we discuss those mechanisms that exist in the thymus and what factors may influence how tolerance is achieved.

Tolerance by Clonal Deletion

Burnet proposed more than 30 years ago that tolerance to self could be achieved by the clonal deletion of autoreactive lymphocytes during development (*9*). A major advance to support this hypothesis came with the development of antibodies to T cell receptor chains with specificity for self antigens. In the first example reported, it was observed that most T cells bearing receptors that contained the β chain of the $V_\beta 17$ family were reactive with the class II MHC

The authors are at the Laboratory of Cellular and Molecular Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.

*To whom correspondence should be addressed.

molecule I-E (*10*). With the use of an antibody that recognizes all $V_\beta17^+$ TCR, T cells bearing this receptor could be observed during development. Although present at the $CD4^+8^+$ stage, such cells are absent from $CD4^+8^-$ and $CD4^-8^+$ thymocytes, as well as peripheral T cells, in mice bearing the I-E self antigen. This finding established clonal deletion as a major mechanism for attaining self tolerance in T cells. Further evidence for clonal deletion was obtained in experiments involving other TCR V_β regions (*11–14*). Clonal deletion has been analyzed also in transgenic mice in which the genes for single TCR α and β chains with defined specificity have been inserted into the germ line. In such mice, the majority of T cells bears a single receptor and are deleted during development in the thymus when their specific antigen is present [(*15*) and von Boehmer (*16*) in this issue].

Critical to understanding the molecular basis of clonal deletion (and other forms of tolerance) is establishing the developmental stage at which this event occurs. At least for some TCRs capable of reacting with class II MHC molecules, both $CD4^+8^-$ and $CD4^-8^+$ cells bearing these receptors are deleted during thymic development. Since class II MHC reactivity is normally associated with $CD4^+$ cells, the deletion also of $CD8^+$ cells bearing such TCRs was unexpected. To account for this, researchers postulated that clonal deletion could occur at the $CD4^+8^+$ precursor stage. Support for this notion came from experiments in which an antibody to CD4 (anti-CD4) was injected in vivo during the course of thymocyte development. Such treatments interfered with CD4 and MHC class II interactions and resulted in the appearance of $V_\beta17^+$ $CD8^+$ T cells in $I\text{-}E^+$ mice (*17*). Since anti-CD4 would not directly affect $CD4^-8^+$ cells, this result indicates that the effect on the $CD4^-8^+$ cells must have occurred at the $CD4^+8^+$ stage. The ability of anti-CD4 to block clonal deletion of potentially self-reactive TCR in $CD8^+$ cells has also been observed for $V_\beta6^+$ TCR, which is specific for a self antigen designated Mls-1^a (minor lymphocyte-stimulating antigen) (*18*). These experiments indicate that clonal deletion requires the participation of the accessory molecules and that deletion does occur at a $CD4^+8^+$ precursor stage.

Experiments involving transgenic mice, in which most T cells bear a receptor specific for a self antigen, also suggest that $CD4^+8^+$ thymocytes are the targets of clonal deletion (*15*). It was shown that in one type of TCRαβ transgenic mouse whose receptor possessed dual specificity for lymphocytic choriomeningitis (LCM) virus or Mls-1^a, clonal deletion of $CD4^+8^+$ thymocytes was much more extensive as a result of LCM virus infection than the presence of the Mls self antigen (*19*). Whether this effect on deletion is the result of the precise developmental stage at which the TCR-bearing cell encounters antigen, the anatomical distribution of the antigen, or some property of presentation peculiar to the specific antigen is unclear. In spite of the fact that the bulk of these studies indicates that clonal deletion occurs at a $CD4^+8^+$ stage, none precludes the possibility that clonal deletion could also occur later at the $CD4^+8^-$ or $CD4^-8^+$ stage.

Clonal deletion does not occur in nude mice (which genetically lack the thymus), which suggests that some component of the thymic environment is an important aspect of this mechanism (*20*). A number of studies have attempted to identify the thymic stromal cells capable of inducing clonal deletion. Thymic stromal cells may be either bone marrow derived (predominantly macrophages or dendritic cells) or epithelial in origin (including both cortical and medullary epithelial cells) (*21*). Most of these cells can express class I and II MHC molecules, allowing them to participate in the clonal deletion process.

To identify stromal cells capable of inducing tolerance, researchers have used chimeric mice to experimentally manipulate the site of MHC antigen expression. Since the epithelial components of the thymus are much more radioresistant than the bone marrow–derived components, radiation can be used to deplete an animal of bone marrow–derived cells while preserving the thymic epithelium. These mice can then be used as a host for the implantation of a bone marrow graft from a mouse with different MHC allelic products (haplotype) (*22*). Thus, the thymus and the developing T cells can differ in their MHC haplotype, but interact within the same animal. In practice, these chimeras are generated by treating recipient mice

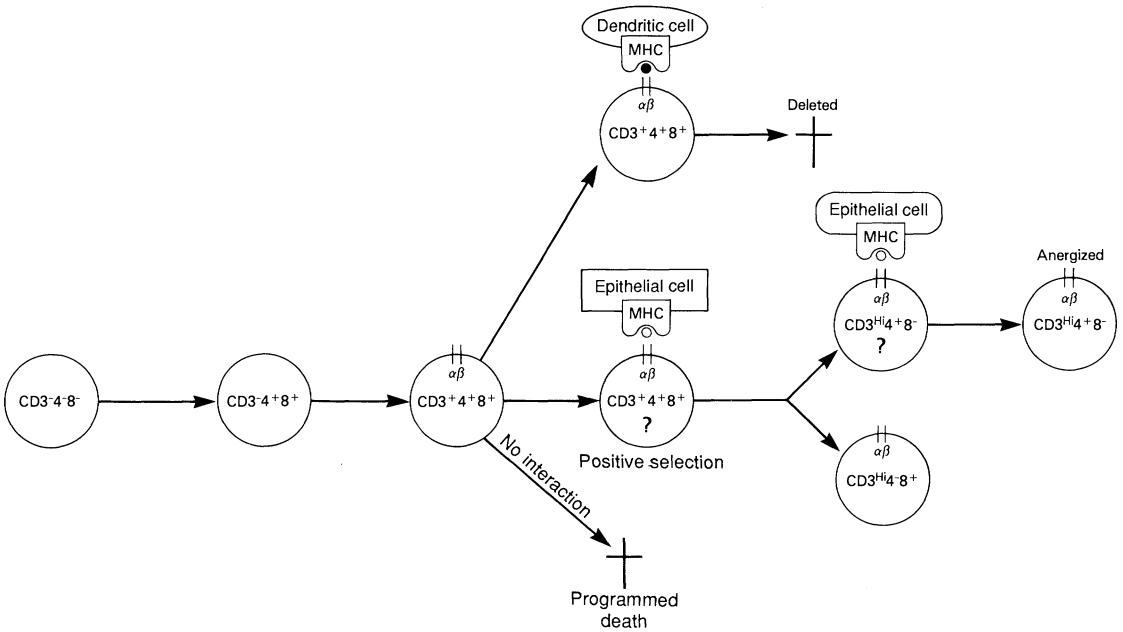


Fig. 1. A model for thymic interactions that could result in clonal anergy. Interactions of $CD4^+8^+$ thymocytes bearing TCRαβ (associated with the invariant CD3 complex) with self MHC plus peptide on dendritic cells or macrophages results in cell death by apoptosis (clonal deletion). Alternatively, a specific interaction of a developing thymocyte with self MHC plus peptide on an epithelial cell (with no subsequent negative selection) results in positive selection (*64*). TCR-bearing thymocytes that fail to bind any ligand die in the thymus by programmed death. It is proposed that if a thymocyte interacts with its ligand on an epithelial cell at a late stage of development, this interaction renders the cell unresponsive to future encounters with its specific antigen (clonal anergy). Some property of this latter interaction must differentiate it from the interaction responsible for positive selection. Here the anergy induction is depicted to occur on a $CD4^+8^-$ cell. This event could occur on either the $CD4^+8^-$ or $CD4^-8^+$ cell if appropriate intracellular signals are delivered. In the absence of such an event in either the thymus or the periphery, these thymocytes can exhibit their full functional potential.

with a high dose of irradiation (1000 R) and then injecting allogeneic or semi-allogeneic bone marrow. Within several weeks, the bone marrow–derived tissues of the host, including the lymphoid system, will be replaced by those of the bone marrow donor. In an alternate chimeric strategy, nude or thymectomized hosts are given allogeneic thymus grafts depleted of bone marrow–derived cells by in vitro culture in deoxyguanosine (*23, 24*).

Other chimeric systems have also been used to identify the cells responsible for inducing self tolerance. Frog and chicken (*25*) chimeras have an advantage in that their generation does not rely on removing the preexisting bone marrow–derived elements (techniques that are subject to problems such as a failure to completely deplete the bone marrow–derived elements or damage to the thymic epithelium). Frog chimeras have been produced by fusing the anterior half of the 24-hour *Xenopus* embryo, containing the thymus anlagen, with the posterior half of an allogeneic embryo, containing bone marrow anlagen, at the same stage of development. A chimera results with thymic epithelial elements expressing one MHC type (encoded by the anterior genotype), while the bone marrow–derived cells express MHC molecules encoded by the posterior genotype. In another system, the thymic rudiment from embryonic chickens is replaced with a xenogeneic, embryonic quail rudiment, producing a chimera with quail thymus epithelium and bone marrow–derived cells of chicken origin. In both of these systems, the chimera is made before the thymus is colonized by stem cells, thus avoiding the need for any type of depletion of bone marrow–derived cells. As will be discussed below, although there is some controversy over the ability of the epithelial components to generate self tolerance in all of these systems, it is nevertheless clear that bone marrow–derived elements are able to induce tolerance.

The initial analysis for self tolerance by a clonal deletion mechanism (with radiation bone marrow chimeras, or thymectomized or thymus-grafting systems) also found that the bone marrow–derived and not the thymic epithelial cells are responsible for clonal deletion (*26*). For example, most of the $V_\beta17^+$ cells were not deleted when I-E was expressed only by the thymic epithelium of the chimeric mice. More recently, transgenic mice have been made by introducing *MHC* genes constructed with controlling elements that target expression to specific cell types (*27–29*). In some cases, mice were produced with transgenic class II MHC molecules expressed selectively in different thymic stromal cells (*27, 29*). When class II I-E was expressed only on the thymic epithelium, the frequency of $V_\beta17^+$ cells was reduced by only 25%, indicating that the majority of the $V_\beta17^+$ cells were not deleted (*26*). In a different transgenic mouse, with I-E expressed on the thymic epithelium, the majority of $V_\beta17^+$ cells were deleted, but the interpretation was complicated by the fact that transgenic I-E was also expressed on some dendritic cells thought to be capable of inducing clonal deletion (*29*). The value of these systems is thus critically dependent on the tissue specificity of transgene expression.

Intrathymic deletion of certain V_β-bearing cells also occurs in mice that possess Mls antigens. Thymocytes bearing $V_\beta8.1$ and $V_\beta6$ TCR are normally eliminated in mice expressing Mls-1^a, whereas $V_\beta3$ TCR^+ cells are eliminated from mice expressing Mls-2^a (*12–15*). Although the *Mls* genes and products are unknown, Mls is the only other endogenous antigen besides the MHC molecules that is able to stimulate in vitro proliferation of a high proportion of T cells from unimmunized Mls^- mice (*30*). Mls antigens appear to differ from conventional antigens in that they can be presented by most MHC molecules (*31*), especially class II I-E; however, there appears to be a hierarchy in the efficiency of Mls presentation by the different mouse MHC haplotypes, in that H-2^k, H-2^d > H-2^b > H-2^q (*12*). The strength of in vitro proliferative responses and the ability to cause deletion appear to reflect this presentation ability. Thus, H-2^k is a permissive haplotype for Mls presentation, whereas H-2^q is a nonpermissive haplotype.

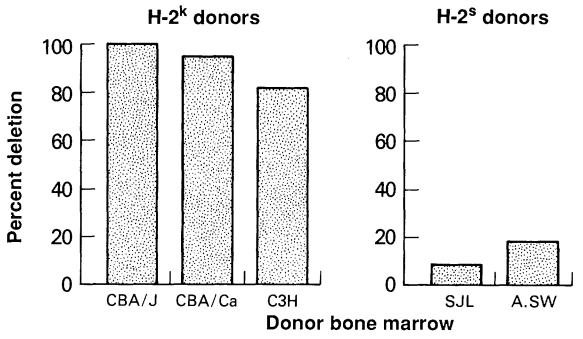
In Mls antigen–mediated clonal deletion, the antigen can be transferred between cells for presentation in vivo (*12*). That is, if radiation chimeras are made with two bone marrow donors (neither of which is able to mediate deletion alone because one expresses Mls but possesses a nonpermissive MHC haplotype for Mls presentation, while the other does not express Mls but is permissive for presentation), the Mls antigen can be transferred from the nonpermissive to the permissive bone marrow–derived cells to mediate deletion. This suggests that at least some antigens in the thymus can be passively acquired. This phenomenon could also account for tolerance induction to antigens found in the circulation and not normally made in the thymus. Mls products are not peculiar in this aspect. Certain minor histocompatibility antigens and the male antigen H-Y appear to share this property in that they are targets for cross-presentation (*32*).

Studies of hybridomas with dual specificity for Mls and antigen reveal that mutations could ablate one specificity without affecting the other (*33*). This led to the suggestion that Mls was recognized by a separate receptor on the T cell independent of the conventional TCR. Since responses to Mls are MHC-dependent, one could argue from this model that the T cell could even recognize Mls and MHC on different presenting cells, thereby forming a tricellular complex. This model could then account for the putative cross-presentation of Mls in vivo. More recent studies, however, have mapped Mls interaction to the conventional TCR, but outside of the antigen-combining site (*34*). The latter data support a model in which the same TCR can recognize an MHC-antigenic complex as well as Mls.

Certain staphylococcal enterotoxins, which stimulate T cells bearing specific V_β families, appear to readily induce clonal deletion of the same TCR^+ thymocytes when administered exogenously (*35*). Antigens, such as these enterotoxins and Mls antigens, that are mitogens for specific V_β families and are able to associate with MHC to mediate clonal deletion of such V_β-bearing T cells irrespective of the α chain have been termed superantigens (*35*). Much speculation exists over whether such V_β mitogens bind MHC molecules for presentation as is envisioned for most conventional antigens. Current evidence suggests that conventional antigens are displayed to T cells only after intracellular proteolytic cleavage and placement into a peptide binding groove on an MHC molecule (*36*). The V_β mitogens, such as Mls and enterotoxins, may bind as uncleaved antigens outside of the binding groove (*37*). In this regard, there is evidence that at least bacterial enterotoxins cannot be presented when cleaved into peptides (*38*).

Tolerance in the Absence of Clonal Deletion

Since it appears that the bone marrow–derived cells mediate clonal deletion, one must account also for tolerance to self antigens expressed by other non–bone marrow–derived tissues both inside and outside the thymus. Variable results have been obtained on the role of the thymic epithelium in the generation of self tolerance. Both the murine thymus engraftment system and the frog or chick-quail chimeras fail to show tolerance to epithelial-type MHC molecules in in vitro functional assays. The commonly used assay is the one-way mixed lymphocyte response (MLR), in which antigen-presenting cells are used to stimulate T cells to proliferate, which is measured by the incorporation of radioactive thymidine into DNA. In spite of the in vitro responses of these chimeras, the thymus graft itself or skin or organ grafts expressing the same MHC antigens are not rejected in vivo (*24, 25*). Thus it would appear that the epithelium has at least some tolerizing capacity. Indeed, tissue-



Donor (bone marrow)		→	Host (epithelium)	
MHC	Mls		MHC	Mls
H-2^k	–		H-2$^{s \times k}$	+
H-2^s	–			

Fig. 2. An analysis for clonal deletion of $V_\beta 6^+$ mature thymocytes in chimeras made with an Mls-1^a–bearing host. The ability to cause deletion is dependent on the transfer of Mls-1^a from host to donor-derived cells and is a function of the *MHC* haplotype of the bone marrow donor. The (B10.S × AKR)F_1 host received 1000 R and was reconstituted with T cell–depleted bone marrow from either H-2^k or H-2^s donors. All donors are *Mls-1^b* except CBA/J, which is *Mls-1^a*. This chimera serves as a control in which the Mls-1^a antigen is expressed by both the donor and host. Data are derived from results published previously (*40*).

specific tolerance may reflect the fact that the actual tolerization step involves not only MHC, but also tissue-specific peptides.

More recently, systems analyzing the clonal deletion of specific V_β families have been used to assess the role of the thymic epithelium in tolerance induction. As mentioned earlier, when the MHC molecule I-E is expressed only on the thymic epithelium, the frequency of $V_\beta 17^+$ T cells is only reduced 10 to 30% (*26, 39–42*). Although most $V_\beta 17^+$ cells are not deleted in these systems, the reduction of $V_\beta 17^+$ cells may reflect some limited deletion induced by the thymic epithelium. It is conceivable that this represents deletion of clones with TCR of high affinity for I-E (*22*).

Irradiation–bone marrow chimeras have also been made in which the Mls antigen is expressed only by the radioresistant host. Clonal deletion can occur in these chimeras by transfer of Mls antigen from the host for presentation by donor bone marrow–derived elements as long as the donor-derived cells express a permissive MHC haplotype (*40, 43*). Clonal deletion is not observed in a variety of bone marrow chimeras in which only the radioresistant host epithelium expresses a permissive MHC type as well as the Mls-1^a antigen. This is illustrated in Fig. 2 for several chimeras, in which (B10.S × AKR)F_1–irradiated recipients (which express MHC H-2$^{s \times k}$) were reconstituted with either H-2^k or H-2^s donor bone marrow. These results show that clonal deletion of $V_\beta 6^+$ T cells occurs only when the donor expresses the permissive, H-2^k haplotype for Mls presentation. Deletion is poor with the nonpermissive H-2^s–expressing donor. Therefore, the ability to cause deletion is a function of the donor bone marrow. Thus, when all the data are considered, the thymic epithelium appears to be relatively poor at inducing clonal deletion.

Given that the majority of $V_\beta 17^+$ and $V_\beta 6^+$ T cells are not eliminated in mice with I-E or Mls-1^a, respectively, expressed only by the radiation-resistant elements, it is appropriate to ask whether these mice are tolerant of the epithelial-type antigens. In transgenic mice that express I-E only on the thymic epithelium and in which the majority of $V_\beta 17^+$ T cells are not deleted, a tenfold reduction is observed in the MLR response to I-E (*26, 39*). In another example, chimeras that fail to delete $V_\beta 6$ (as shown in Fig. 2) or $V_\beta 17$ show a drastic reduction in their proliferative response directed toward antigens of host (epithelial) type, even though these chimeras respond vigorously to controls (*40, 42*). These data reveal that donor bone marrow cells that have developed within a semi-allogeneic host thymus can be tolerized to host antigens even if they are not deleted.

These results are in contrast to those obtained in certain murine radiation bone marrow chimeras, thymus-grafted chimeras, and in the frog or chick-quail chimeras (*23, 24, 44*). From such systems, it appears that in vitro mixed lymphocyte or cytolytic responses were obtained. Whether the differences in the in vitro responses between these chimeras and those described previously are due to quantitative or qualitative aspects of antigen expression in the experimental system is not clear. Certainly, multiple mechanisms of tolerance induction may operate and may vary for the two T cell lineages (CD4 versus CD8) or as a result of the tolerizing tissue (caused by differential signaling or tissue-specific peptides).

The presence in the bone marrow chimeras of T cells bearing V_β segments ($V_\beta 6$ and $V_\beta 17$), which correlates with responsiveness to Mls-1^a and I-E and the absence of an MLR to the relevant antigens, suggests that tolerance is achieved by clonal anergy. That is, these specific TCR-bearing clones appear to be functionally inactivated. This was demonstrated at the clonal level by stimulating with V_β-specific plate-bound antibodies. These antibodies fail to stimulate $V_\beta 6^+$ or $V_\beta 17^+$ mature thymocytes from the chimera, even though other T cells in the population respond well to a control stimulation with an antibody to TCR$\alpha\beta$ (*40, 42*). The failure to respond was observed over a broad dose range of antibody. It is significant that both mature thymocytes and peripheral T cells manifested comparable levels of tolerance. Thus, thymic tolerance can also result from specific clonal anergy.

In the chimeras shown in Fig. 2, it was concluded that Mls could be transferred in vivo from host to donor and that clonal deletion was determined by the nature of the bone marrow–derived cells. By isolating the expression of Mls antigens and MHC molecules in bone marrow chimeras, it is possible to address the question of whether Mls can be transferred in the opposite direction, for example, from donor to host cells. Any relevant presentation of Mls on a permissive *MHC* haplotype would presumably be localized to the radioresistant thymic epithelium.

Table 1 summarizes the results obtained with various chimeric combinations of *Mls* and *MHC* gene expression by bone marrow–derived and thymic epithelial cells with respect to their ability to induce clonal deletion and clonal anergy. Data from this and other chimeric systems indicate that the self antigen Mls can be transferred for presentation in vivo (*12, 40, 43*). Deletion of Mls-reactive T cells occurs only if the bone marrow expresses a permissive MHC for presentation, regardless of the source of Mls antigen. If, however, the radioresistant host expresses Mls and bears a permissive *MHC* haplotype, tolerance is induced via clonal anergy. In contrast, it appears that Mls expressed by the donor cannot be transferred to permissible MHC molecules on the host to induce anergy (*45*). This latter system relied on the reactivity of $V_\beta 3^+$ T cells for the Mls-2^a antigen. The failure to anergize these $V_\beta 3^+$ cells could be because of a failure to efficiently transfer Mls in the opposite direction (donor → host) for presentation by the epithelium, or because Mls-1^a is a stronger antigen than Mls-2^a for mediating interactions that induce clonal anergy. Previous studies, which show that Mls-2^a can mediate clonal deletion of $V_\beta 3^+$ cells in mixed bone marrow chimeras, demonstrate that Mls-2^a transfer, per se, is possible (*12*). The source of Mls antigen in such chimeras has not been determined. Experiments with deoxyguanosine-depleted thymus grafts have shown that the thymic epithelium itself is unable to produce the Mls antigen

Table 1. A summary of tolerance induction by clonal deletion or clonal anergy in $V_\beta 6^+$ T cells in P → F_1, irradiation bone marrow chimeras.

Donor (bone marrow–derived)		Host (epithelium)		Deletion	Anergy
Mls	MHC	Mls	MHC		
+	Permissive	+	Permissive	Yes	
−	Permissive	+	Permissive	Yes	
−	Permissive	+	Nonpermissive	Yes	
−	Nonpermissive	+	Permissive	No	Yes

(*45, 46*). This does not preclude the possibility, however, that the thymic epithelium can present transferred Mls.

The results in Table 1 could also be interpreted in another way to explain why deletion versus anergy is obtained. Rather than invoking different inducing cell types for these different forms of tolerance, one could argue that all the tolerance is induced by the bone marrow–derived elements. In this model, the nonpermissible MHC molecules are able to present Mls, but do so at a quantitatively lower level. Thus, a high level of receptor occupancy leads to clonal deletion, whereas a low level results in clonal anergy.

Induction of Clonal Anergy by the Thymus

Defining the various components of TCR-MHC-ligand interactions, the interacting cell types, the intracellular signals, and the stage of development are critical to understanding the mechanism involved in tolerance induction. Figure 1 shows how self tolerance may be generated in a developmental model. The particular interaction of the TCRαβ with MHC molecules (or MHC plus peptide) in the thymic microenvironment determines the fate of the cell bearing that receptor. Thus, an interaction with some element of the thymic epithelium (in the absence of any negative selection) leads to functional maturity. An interaction with a bone marrow–derived macrophage or dendritic cell induces death, that is, clonal deletion. Since these different destinies for a developing T cell involve the same TCR and MHC, there must be other variables that determine these two developmental outcomes. One possibility is that the developmental stage of the T cell could be important in these events. Thus, TCR and MHC interactions occurring at an early stage of maturation may result in a different developmental fate from a similar interaction occurring at a later stage of maturation (*47*). There are a number of features of the T cell that may affect signal transduction that are known to change during development. Such parameters include the surface expression and participation of accessory molecules, such as CD4 and CD8, the cell surface density of the TCR, the type of TCR-associated signaling components, the inducibility of specific genes, and the nature of the intracellular biochemical pathways. The other possibility is that the stromal cells that bear a ligand recognized by the TCR (either for positive or negative selection) are different and therefore may display unique peptides (*1*), accessory or adherence molecules, or deliver other necessary signals. In support of this latter possibility, it is known that the thymic epithelial cell is involved in inducing positive selection, whereas the bone marrow–derived dendritic cell or macrophage is involved in clonal deletion.

An in vitro model for inducing anergy in mature T cell clones has been put forth by Jenkins and colleagues (*48*). In this model, $CD4^+$ class II–specific T cells are activated for proliferation only when the TCR is occupied in the presence of a second or costimulatory signal. When signal one, that is, peptide plus MHC molecules, is presented in the absence of the second signal, these clones do not proliferate and, moreover, become unresponsive to subsequent stimulation by cells capable of providing both signals, that is, they are anergized. This one versus two signal model is therefore similar to that first put forth by Bretscher and Cohn to account for tolerance in B cells (*49*). Whereas this system has been established for class II–dependent T cells, differential signaling events may also account for tolerance in class I–dependent cells (*50*).

Clonal anergy in the thymus could result then from the delivery of only one signal through occupancy of the TCR in the absence of costimulation. Since $V_\beta 6^+$ and $V_\beta 17^+$ T cells are anergized in the chimeric thymus when the relevant antigens were presented on the radioresistant host (*40, 42*), a likely candidate for inducing clonal anergy in that case is the thymic epithelium. By this hypothesis, the epithelial cell (if it exhibits the relevant antigen-MHC complex) is able to provide only one signal. Supporting this idea, thymic epithelial cells express MHC molecules and can present antigen to T cell hybridomas, but are unable to activate T cell clones (*51*). Heterogeneity of thymic epithelial cells is a potential added complication, as anergy induction may be a function of only a subset of epithelial cells.

The epithelial model for anergy induction should also be considered in the developmental scheme. At what T cell stage is the anergy induced? Isolated subsets of both $CD4^+8^-$ and $CD4^-8^+$ thymocytes and peripheral T cells from tolerized chimeras have been found to be anergic (*45*). This result is somewhat surprising, since both the $V_\beta 17$ and $V_\beta 6$ responses are class II–dependent and are generally limited to $CD4^+8^-$ cells. If, however, anergy induction occurs at a $CD4^+8^+$ precursor stage, where CD4 is available for all receptors, anergy might be expected to occur in both subsets. To address this question we performed in vivo anti-CD4 treatments on newly constructed chimeras to determine whether anergy induction could be inhibited. These treatments, however, failed to affect the anergy of either the $CD4^-8^+$ thymocytes or peripheral T cells (*45*). This result is in contrast to previous studies showing that anti-CD4 treatments are able to reverse clonal deletion of $V_\beta 17^+$ thymocytes [(*17*) and above]. Whereas the effect on anergy is a negative result and not conclusive, the data suggest that the anergy induction does not occur at the $CD4^+8^+$ precursor stage or that CD4 is not used in the interaction that leads to this form of tolerance. If CD4 is not used in this interaction, anti-CD4 treatment cannot be used to stage the anergy induction event.

If anergy of $V_\beta 6^+$ and $V_\beta 17^+$ clones can be induced in the $CD4^-8^+$ thymocytes, then one would have to conclude that these cells are capable of recognizing class II without CD4. Although this concept is counter to the prevailing dogma that responses by $CD8^+$ cells are predominantly class I dependent, it may have some validity. Several lines of evidence support the notion that $CD8^+$ T cells can interact with certain class II–dependent antigens. It has been shown that $CD8^+$ $V_\beta 11^+$ T cells respond to I-E in vivo, although not in vitro (*52*). In general, the nature of the I-E–dependent responses and deletion of $V_\beta 11$-bearing cells is typical of TCRs that recognize antigens that are V_β mitogens. Also supporting this concept is the fact that $CD8^+$ cells can respond to enterotoxins, which are typically class II–dependent antigens (*53*). In transgenic mice in which I-E is expressed only on the pancreatic islet cells, both $CD8^+$ and $CD4^+$ $V_\beta 17^+$ T cells are anergized, also indicating that $CD8^+$ cells can recognize class II I-E in vivo (*41*). Thus, unlike conventional responses, $CD8^+$ cells bearing V_β-reactive TCR may interact with a sufficiently high avidity to mediate anergy without the aid of accessory molecules. In this regard, it would be of interest to determine if $CD8^+$ cells bearing the class I–restricted, H-Y–reactive TCR in transgenic mice could be anergized if the antigen is expressed only on the thymic epithelium, since H-Y is not a V_β mitogen.

Another issue to consider in the model shown in Fig. 1 is why the interaction that induces clonal anergy does not instead induce clonal deletion. For clonal deletion, it is possible to invoke the two-signal model postulated for T cell activation. As discussed above, it is conceivable that clonal deletion can only be induced in cells during an early stage of T cell development. One could argue that two signals induce clonal deletion in an immature T cell, whereas these same signals promote activation in a mature T cell. In support of this concept, it has been shown that splenic dendritic cells (which are capable of providing a costimulatory signal to mature T cells) will induce tolerance when added to fetal thymic organ cultures (*54*). Although this tolerance has been measured in a functional assay and clonal deletion per se could not be examined, the expectation would be that such (bone marrow–derived) antigen-presenting cells are causing clonal deletion. Thus, an interaction delivering only signal one (occupancy of the TCR) may be insufficient to induce clonal deletion in immature thymocytes and might instead induce clonal anergy.

The model presented in Fig. 1 raises another problem. If all cells that are positively selected on the epithelium could also be anergized by the same epithelium, all mature T cells would be nonfunctional. There are two ways out of this dilemma. (i) There is evidence for cellular heterogeneity in the thymic epithelium. Medullary epithelial cells can be distinguished from cortical epithelial cells with monoclonal antibodies (*55*). Thus, different epithelial components could be involved in positive selection as opposed to anergy induction. (ii) Positive selection and anergy induction may operate at different stages of T cell ontogeny. Thus, distinct cells may be capable of inducing distinct differentiation events, T cell signaling may vary during development, or both.

If thymic tolerance by clonal anergy does occur at a late stage of thymic development as depicted in Fig. 1, it may not be any different than the anergy proposed to occur in mature peripheral T cells (*56, 57*). In two in vivo systems, mature cells bearing Mls-reactive TCRs were rendered tolerant to transferred Mls^+ cells. In one case, the tolerized $V_\beta6^+$ cells expressed interleukin-2 (IL-2) receptors in vivo, suggesting they were responding to antigen, but they were unable to produce IL-2 in response to Mls-1^a in vitro (*57*). The anergized cells from bone marrow chimeras also made a partial response in that they expressed some IL-2 receptors upon stimulation (*40, 42*), although at a lower level and frequency than pseudochimeric controls. Therefore, they could respond to antibodies to TCR in the presence of exogenously added IL-2, although never to the level of the controls. Thus, it would appear that a major defect in the anergized cells is an inability to make IL-2. This result is similar to that obtained from the in vitro anergy system described above (*48*). Interestingly, anergized clones in the chimeras appeared not to be responding to antigen in vivo, in that they were no larger than other T cells and did not express IL-2 receptors (*45*). As is true for the peripheral tolerance models, mixing responsive and nonresponsive cells has failed to show any suppression by the anergized cells. Thus, the chimeric system described is able to reveal a thymically induced self tolerance in the form of clonal anergy. The only difference between thymic and peripheral anergy would therefore be the tissue responsible for inducing the tolerance. Each cell type, the thymic epithelial cell, or the islet or acinar cell (in the case of I-E transgenic mice), must be unable to induce the second signal.

Still another possibility exists. During the $CD4^+8^-$ or $CD4^-8^+$ stage of development, the T cell may encounter its antigen on a conventional antigen-presenting cell, that is, one that could deliver two signals but, because of the immature state of the T cell, it does not yet express receptors for the second (costimulatory) signal. In such a case, anergy rather than activation may result.

Significance and Future Directions

Although self tolerance can be achieved in the thymus by induction of clonal anergy in radiation chimeras, the question is raised as to whether this mechanism exists in normal physiological settings. The chimeras discussed were constructed in such a way as to prevent clonal deletion and thereby reveal clonal anergy. Whereas only nonchimeric systems can resolve this issue, there is good evidence for induction of clonal anergy in the periphery, and it seems reasonable that the same mechanism would operate in the thymus.

It is possible also that other forms of tolerance may yet be uncovered. As mentioned earlier, a third type of tolerance could involve suppression. Although there is good evidence that regulatory mechanisms may operate that are probably anti-idiotypic in nature (*58, 59*), there is yet no clear evidence that the thymus participates in these forms of tolerance.

There may be other thymic tolerance mechanisms. Some $TCR\alpha\beta$ $CD4^-8^-$ cells that bear "forbidden" TCRs [TCRs that are normally deleted during development (*60*)] are generated in the thymus. Such cells could be produced by a thymic interaction that causes the down-regulation of accessory molecules or TCR, or both. This could render the cells less reactive, since both the appropriate accessory molecule (CD4 or CD8) and TCR are necessary to maintain the reactivity of some receptors (*61*). By functional analysis, $TCR\alpha\beta$ $CD4^-8^-$ thymocytes are not reactive to self.

At face value, it appears that tolerance induction by clonal anergy could serve as an important alternative to clonal deletion. The thymic epithelium could render T cells anergic to epithelial-specific peptides that may be expressed by peripheral tissues but are not expressed by thymic dendritic cells. The suggestion has also arisen that an anergized but nondeleted T cell may serve also as a specific suppressor, because it can bind to antigen and perhaps consume lymphokines while being unable itself to produce lymphokines or to respond by proliferation (*62, 63*). In this way, the anergized cell would dampen an ongoing immune response by competing for antigen and lymphokines. It is possible that from the $CD4^+8^-$ or $CD4^-8^+$ thymocyte stage, and for a finite time after export from the thymus, T cells may be more easily tolerized by clonal anergy. This would allow newly emerging T cells to be rendered nonresponsive to the many self antigens not found in the thymus. If such a mechanism existed, it could account for the failure to tolerize exogenously transferred mature T cells with transgenic I-E expressed on pancreatic acinar cells in the same animals in which endogenous T cells are I-E–tolerant (*63*). The implication from the latter data is that not all peripheral T cells can be clonally inactivated according to the one-signal model of anergy induction. The demonstration of clonal anergy as another mechanism for maintaining self tolerance may account for nonresponsiveness to antigens present only in peripheral tissues or present in the thymus at insufficient levels to cause clonal deletion. Since the induction of clonal anergy may be used clinically to prevent graft rejection as well as to abort ongoing autoimmune processes, it is now important to elucidate the conditions under which these various forms of tolerance may be induced and maintained.

REFERENCES AND NOTES

1. P. Marrack and J. Kappler, *Science* **238**, 1073 (1987).
2. D. H. Raulet, *Annu. Rev. Immunol.* **7**, 175 (1989).
3. A. Weiss *et al.*, *ibid.* **4**, 593 (1986).
4. C. Doyle and J. L. Strominger, *Nature* **330**, 256 (1987); A. M. Norment, R. D. Salter, P. Parham, V. H. Engelhard, D. R. Littman, *ibid.* **336**, 79 (1988).
5. J. D. Marth, R. Peet, E. G. Krebs, R. M. Perlmutter, *Cell* **43**, 393 (1985); A. Veillette, M. A. Bookman, E. M. Horak, J. B. Bolen, *ibid.* **55**, 301 (1988); A. Veillette, M. A. Bookman, E. M. Horak, L. E. Samelson, J. B. Bolen, *Nature* **338**, 257 (1989).

6. E. Robey, B. J. Fowlkes, D. Pardoll, *Semin. Immunol.*, in press.
7. M. Blackman, J. W. Kappler, P. Marrack, *Science* **248**, 1335 (1990).
8. G. A. Dos Reis and E. M. Shevach, *J. Exp. Med.* **157**, 1287 (1983); E. S. Groves and A. Singer, *ibid.* **158**, 1483 (1983); H.-G. Rammensee and M. J. Bevan, *Nature* **308**, 741 (1984); P. Matzinger, R. Zamoyska, H. Waldmann, *ibid.*, p. 738.
9. F. M. Burnett, *The Clonal Selection Theory of Acquired Immunity* (Cambridge Univ. Press, New York, 1959).
10. J. W. Kappler, N. Roehm, P. Marrack, *Cell* **49**, 273 (1987).
11. J. W. Kappler, U. Staerz, J. White, P. C. Marrack, *Nature* **332**, 35 (1988); H. R. MacDonald *et al.*, *ibid.*, p. 40.
12. A. M. Pullen, P. Marrack, J. W. Kappler, *ibid.* **335**, 796 (1988).
13. R. Abe, M. S. Vacchio, B. Fox, R. J. Hodes, *ibid.*, p. 827; A. M. Fry and L. A. Matis, *ibid.*, p. 830.
14. J. Bill, O. Kanagawa, D. L. Woodland, E. Palmer, *J. Exp. Med.* **169**, 1405 (1989).
15. P. Kisielow, H. Blüthmann, U. D. Staerz, M. Steinmetz, H. von Boehmer, *Nature* **333**, 742 (1988); W. C. Sha *et al.*, *ibid.* **336**, 73 (1988); L. J. Berg, B. F. de St. Groth, A. M. Pullen, M. M. Davis, *ibid.* **340**, 559 (1989).
16. H. von Boehmer and P. Kisielow, *Science* **248**, 1369 (1990).
17. B. J. Fowlkes, R. H. Schwartz, D. M. Pardoll, *Nature* **334**, 620 (1988).
18. H. R. MacDonald, H. Hengartner, T. Pedrazzini, *ibid.* **335**, 174 (1988).
19. H. Pircher *et al.*, *ibid.* **342**, 559 (1989).
20. A. M. Fry, L. A. Jones, A. M. Kruisbeek, L. A. Matis, *Science* **246**, 1044 (1989); R. J. Hodes, S. O. Sharrow, A. Solomon, *ibid.*, p. 1041.
21. A. N. Barclay and G. Mayrhofer, *J. Exp. Med.* **153**, 1666 (1981); E. J. Jenkinson, W. Van Ewijk, J. J. T. Owen, *ibid.*, p. 280; B. Adkins *et al.*, *Annu. Rev. Immunol.* **5**, 325 (1987).
22. J. Sprent, D. Lo, E.-K. Gao, Y. Ron, *Immunol. Rev.* **101**, 173 (1988).
23. E. J. Jenkinson, L. L. Franchi, R. Kingston, J. J. T. Owen, *Eur. J. Immunol.* **12**, 583 (1982).
24. A. R. Ready, E. J. Jenkinson, R. Kingston, J. J. T. Owen, *Nature* **310**, 231 (1984); H. von Boehmer and K. Schubiger, *Eur. J. Immunol.* **14**, 1048 (1984).
25. M. F. Flajnik, L. DuPasquier, N. Cohen, *ibid.* **15**, 540 (1985); H. Ohki, C. Martin, C. Corbel, M. Coltey, N. M. Le Douarin, *Science* **237**, 1032 (1987).
26. P. Marrack *et al.*, *Cell* **53**, 627 (1988).
27. G. Widera *et al.*, *ibid.* **51**, 175 (1987).
28. J. Allison *et al.*, *Nature* **333**, 529 (1988); A. Dorn *et al.*, *Mol. Cell. Biol.* **8**, 3975 (1988).
29. W. Van Ewijk *et al.*, *Cell* **53**, 357 (1988).
30. H. Festenstein, S. Kumura, G. Biasi, *Immunol. Rev.* **107**, 29 (1989); R. Abe and R. J. Hodes, *Annu. Rev. Immunol.* **7**, 683 (1989).
31. C. A. Janeway, Jr., K. Fischer-Lindahl, U. Hammerling, *Immunol. Today* **9**, 125 (1988).
32. R. D. Gordon, B. J. Mathieson, L. E. Samelson, E. A. Boyse, E. Simpson, *J. Exp. Med.* **144**, 810 (1976); R. Korngold and J. Sprent, *ibid.* **151**, 314 (1977); P. Matzinger and M. H. Bevan, *Cell Immunol.* **33**, 92 (1977); H. von Boehmer and K. Hafen, *Nature* **320**, 626 (1986).
33. S. R. Webb, A. Okamoto, J. Sprent, *J. Immunol.* **141**, 1828 (1988).
34. A. Pullen, T. Wade, P. Marrack, J. Kappler, personal communication.
35. J. White *et al.*, *Cell* **56**, 27 (1989).
36. P. J. Bjorkman *et al.*, *Nature* **329**, 506 (1987); P. J. Bjorkman *et al.*, *ibid.*, p. 512; E. R. Unanue and P. M. Allen, *Science* **236**, 551 (1987).
37. C. A. Janeway, Jr., *et al.*, *Immunol. Rev.* **107**, 61 (1989).
38. J. D. Fraser, *Nature* **339**, 221 (1989).
39. D. Lo, L. C. Burkly, R. A. Flavell, R. D. Palmiter, R. L. Brinster, *Semin. Immunol.* **1**, 147 (1989).
40. F. Ramsdell, T. Lantz, B. J. Fowlkes, *Science* **246**, 1038 (1989).
41. L. C. Burkly, D. Lo, O. Kanagawa, R. L. Brinster, R. A. Flavell, *Nature* **342**, 564 (1989).
42. J. L. Roberts, S. O. Sharrow, A. Singer, *J. Exp. Med.* **171**, 935 (1990).
43. D. E. Speiser, R. Schneider, H. Hengartner, H. R. MacDonald, R. M. Zinkernagel, *ibid.* **170**, 595 (1989); Y. Yoshikai *et al.*, *Eur. J. Immunol.* **19**, 1009 (1989); D. E. Speiser, R. K. Lees, H. Hengartner, R. M. Zinkernagel, H. R. MacDonald, *J. Exp. Med.* **170**, 2165 (1989).
44. J. Sprent, H. von Boehmer, M. Nabholz, *J. Exp. Med.* **142**, 321 (1975).
45. F. Ramsdell and B. J. Fowlkes, in preparation.
46. H. Yuuki *et al.*, *J. Immunol.* **144**, 474 (1990).
47. J. Lederberg, *Science* **129**, 1649 (1959).
48. M. K. Jenkins, D. M. Pardoll, J. Mizuguchi, T. M. Chused, R. H. Schwartz, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5409 (1987); D. L. Mueller, M. K. Jenkins, R. H. Schwartz, *Annu. Rev. Immunol.* **7**, 445 (1989); R. H. Schwartz, *Science* **248**, 1349 (1990).
49. P. Bretscher and M. Cohn, *Science* **169**, 1042 (1970).
50. S. Guerder and P. Matzinger, in *Quantitative Biology Symposia* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, in press); M. A. Rus, A. S. Rosenberg, T. I. Munitz, A. Singer, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2765 (1990); G. Otten and R. Germain, personal communication.
51. R. G. Lorenz and P. M. Allen, *Nature* **340**, 557 (1989).
52. E.-K. Gao, O. Kanagawa, J. Sprent, *J. Exp. Med.* **170**, 1947 (1989).
53. B. Fleischer and H. Schrezenmeier, *ibid.* **167**, 1697 (1988).
54. P. Matzinger and S. Guerder, *Nature* **338**, 74 (1989).
55. E. van Vliet, E. J. Jenkinson, R. Kingston, J. J. T. Owen, W. Van Ewijk, *Eur. J. Immunol.* **15**, 675 (1985).
56. S. Qin, S. Cobbold, R. Benjamin, H. Waldmann, *J. Exp. Med.* **169**, 779 (1989).
57. H.-G. Rammensee, R. Kroschewski, B. Frangoulis, *Nature* **339**, 541 (1989).
58. D. B. Wilson, *Immunol. Rev.* **107**, 159 (1989).
59. C. A. Janeway, *Nature* **341**, 482 (1989).
60. B. J. Fowlkes and D. M. Pardoll, in *Advances in Immunology*, F. J. Dixon, Ed. (Academic Press, New York, 1989), vol. 44, pp. 207–264; H.-S. Teh, H. Kishi, B. Scott, H. von Boehmer, *J. Exp. Med.* **169**, 795 (1989); M. Egerton and R. Scollay, *Int. Immunol.* **2**, 157 (1990); D. M. Pardoll and B. J. Fowlkes, in preparation.
61. Z. Dembić *et al.*, *Nature* **326**, 510 (1987); J. Gabert *et al.*, *Cell* **50**, 545 (1987).
62. J. Gunther, W. Haas, H. von Boehmer, *Eur. J. Immunol.* **12**, 247 (1982).
63. D. Lo, L. C. Burkly, R. A. Flavell, R. D. Palmiter, R. L. Brinster, *J. Exp. Med.* **170**, 87 (1989).
64. D. Lo and J. Sprent, *Nature* **319**, 672 (1986); P. Kisielow, H. S. Teh, H. Blüthmann, H. von Boehmer, *Nature* **335**, 730 (1988); L. J. Berg *et al.*, *Cell* **58**, 1035 (1989).

65. The authors gratefully acknowledge R. H. Schwartz for continued advice and support; B. R. Marshall for expert editorial assistance; and R. Germain, E. Robey, D. Pardoll, M. Lenardo, J. Allison, and E. Fuchs for critical reading of the manuscript.