Brief Definitive Report

Evidence That Binding Site Occupancy Is Necessary and Sufficient for Effective Major Histocompatibility Complex (MHC) Class II Transport through the Secretory Pathway Redefines the Primary Function of Class II–associated Invariant Chain Peptides (CLIP)

By Guangming Zhong, Flora Castellino, Paola Romagnoli, and Ronald N. Germain

From the Lymphocyte Biology Section, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-1892

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Summary

Invariant chain (Ii) associates with newly synthesized class II molecules in the endoplasmic reticulum (ER), an interaction that has been shown to interfere with peptide binding to class II molecules. The class II–associated invariant chain peptide (CLIP) region (residues 81–104) of Ii is believed to mediate this inhibition by engaging the binding domain of class II like an antigenic peptide. Together, these findings have given rise to a model in which CLIP association with the class II groove acts to prevent inappropriate presentation of peptides imported into the ER for association with major histocompatibility complex class I molecules. However, the properties of class II molecules synthesized by cells lacking coexpressed Ii are at least superficially inconsistent with this paradigm in that they do not show clear evidence of peptide acquisition. At the same time, we have previously shown the shortest form of Ii still containing CLIP to play an essential role in regulation of early class II molecule assembly and transport in the secretory pathway. Using covalent peptide technology, we now show that occupancy of the class II binding site in the ER regulates class II trafficking to the Golgi complex, an event that is the locus of the major defect in cells of Ii-deficient mice. These data argue that CLIP occupies the class II binding site, not to prevent interaction with short peptides meant for class I, but rather to maintain the structural integrity of class II molecules that are labile without engaged binding regions, and that would also associate with intact proteins in the ER if left unoccupied. By these means, CLIP occupancy of the class II binding site promotes effective export of useful class II molecules for endocytic peptide acquisition.

Invariant chain (Ii) regulates many of the properties of MHC class II molecules (1), including peptide binding in vitro (2, 3). This latter observation led to the view that the class II–associated invariant chain peptide (CLIP) region of Ii (residues 81–104) occupies the class II binding site (4) to inhibit presentation of the endogenous peptides that normally associate with MHC class I molecules in the endoplasmic reticulum (ER) (5, 6). Newly synthesized class II molecules from cells lacking Ii, however, do not show clear evidence of the peptide binding that is predicted by this model. Class II proteins synthesized without Ii undergo inefficient α/β chain assembly in some haplotypes (7–9), fail to achieve an SDS-stable conformation, transport poorly from ER to the Golgi complex (7–10), aggregate (11), and do not show tight association with elutable peptide ligands (12).

Intriguingly, CLIP makes a critical contribution to preventing all of these defects in early class II assembly and transport, especially class II ER-to-Golgi complex trafficking (13). Unoccupied class II molecules are known to rapidly denature or misfold (14–16), and they can bind to intact proteins (17, 18), events that would be likely to inhibit effective postsynthetic transport of class II molecules. Together, these observations led us to consider that any inhibition of ER peptide binding by CLIP interaction with the class II groove might be incidental to a required role in establishing and preserving a functional class II conformation that promotes efficient transport (1, 13, 19). In agreement with this hypothesis, we report here that class II occupancy in the ER by either short antigenic peptides or by CLIP alone fully replaces the secretory pathway functions of Ii, emphasizing the concept that class II activity depends on controlled engagement of the binding region at all times after synthesis (19).

Materials and Methods

cDNA Expression Constructs. The cDNA constructs coding for the wild-type α or β chain of A^k and A^b in plasmid CDM8 and

the cDNA coding for murine Ii31 and Ii19-107 in plasmid pcEXV-3 have been described previously (13, 20). The Eα52-68: $A\beta^b$ construct in plasmid pHbAPR-1-neo was kindly provided by Drs. J. Kappler and P. Marrack (HHMI, Denver, CO) (21). The constructs hen egg lysozyme (HEL)116-129:$A\beta^k$, HEL46-61: $A\beta^k$, HEL46-61mut:$A\beta^k$, and mclip:$A\beta^k$ in plasmid pCDL-SRα (22) were generated in the following manner. The HEL46-61:$A\beta^k$ construct was prepared first and served as a cloning vector for making the other $A\beta^k$ constructs. Two partially overlapping oligos were synthesized with the forward primer sequence 5′-ACTGAG-GGCGGAAACTCCGGCCCAGGAGGCCGTAACACCGAT-GGGAGTACCGACTACGGAATCCTACAGATCAACAGCA-GGCCG-3′ and the backward primer sequence 5′-CTGGTGC-ACGAAATGCCTTTCGGATCCACCTCCACCTGGTGCT-GAGCCACCGCCTCCAGGCCCTGACGGCCTGCTGTTG-ATCTG-3′. The partially overlapping primers were extended using Taq polymerase to produce a double stranded DNA fragment that codes for HEL peptide 46-61 and a linker region. The DNA fragment was then inserted in between codons 4 and 5 of β1 domain of mature $A\beta^k$. Two noncompatible sfiI sites were placed flanking the peptide coding region, allowing the enclosed peptide-coding sequence to be replaced by other peptide-encoding sequences. To make the HEL116-129:, HEL46-61mut:, and CLIP: $A\beta^k$ constructs, dsDNAs coding for the corresponding peptides were made by extending two partially overlapping oligos using PCR and then splicing them into the HEL46-61:$A\beta^k$-pCDL-SRα plasmid by using the sfiI sites. As a result, the HEL46-61 was replaced with HEL116-129, HEL46-61mut, or mouse CLIP (see Fig. 1 for amino acid sequences). The HEL46-61mut sequence was derived from HEL46-61 and contains six amino acid substitutions: D52 to R, Y53 to A, L56 to G, Q57 to A, 58I to K, and 61R to G. These residues are either A^k or TCR contact residues (23). The sequences were confirmed using an automated DNA sequencer.

Transfection. COS 7.2 cells were transiently transfected with cDNA constructs using a DEAE–dextran method as previously described (24) and incubated 48 h in DMEM/10% FCS. Rat basophil leukemia (RBL) cells were stably transfected using electroporation followed by selection in G418 (Geneticin; GIBCO BRL, Gaithersburg, MD). G418-resistant cells were analyzed by flow cytofluorometry 7–10 d after transfection. Positive cells were cloned by limiting dilution. The clones with intermediate expression level were used in the experiments.

Metabolic Labeling, Immunoprecipitation, and SDS-PAGE. Metabolic labeling, immunoprecipitations, and SDS-PAGE were carried out as described elsewhere (14, 25) with some modifications. The class II molecules were precipitated with the mAb M5/114 (26), the mouse mAb Y-Ae (specific for the A^b molecule bound to the Eα52-68 peptide [27]), or the mAb 10.2.16 (28) (specific for $A\beta^k$), all previously bound to protein G–Sepharose or protein–A Sepharose beads. Where samples were examined for SDS-stable, compact dimers, half of the precipitate was eluted using sample buffer containing 2% SDS and 5% 2-ME without heating; the other half was boiled in sample buffer (14, 15). Both eluates were then analyzed using 10% polyacrylamide gels. For analysis of N-linked glycan status, the precipitate was eluted with 2% SDS in PBS by boiling for 5 min. The eluate was diluted 10-fold with lysis buffer and then reprecipitated with a rabbit antiserum raised against a peptide corresponding to a sequence from the tail of class II α chains (25). The reprecipitate was divided into two parts and incubated in 50 mM sodium citrate buffer, pH 5.5, in the presence or absence of 15 mU of endoglycosidase H (endo H; Boehringer Mannheim Corp., Indianapolis, IN) at 37°C for 16 h (29). The treated samples were then eluted using normal SDS-PAGE sample buffer with heating and analyzed on 10% gels.

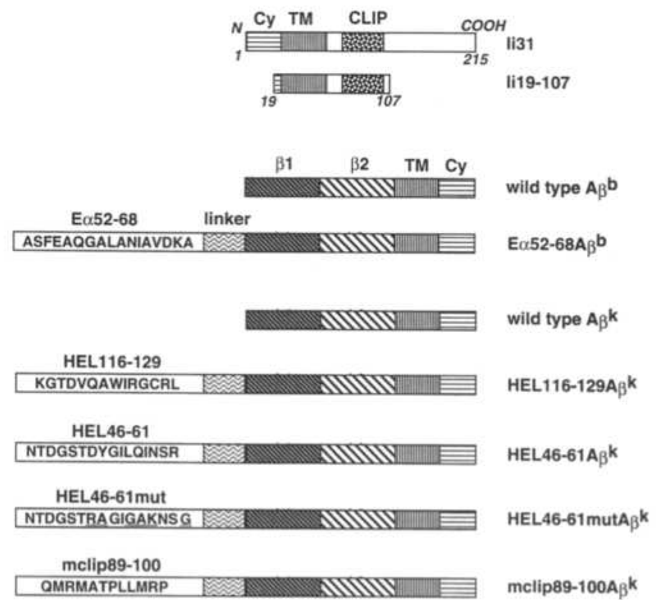

Figure 1. Structure of Ii and class II wild-type and variant proteins. The protein structures of full-length mouse Ii (p31), the truncated membrane-anchored form Ii19-107 containing CLIP, wild-type $A\beta^b$ and $A\beta^k$, peptide-tethered $A\beta^b$ and $A\beta^k$, and wild-type $A\alpha^b$ and $A\alpha^k$ are illustrated diagrammatically.

Results

Rapid Binding of Tethered Peptides to the Class II Binding Groove. To examine whether groove binding could substitute for Ii in promoting class II ER to Golgi complex trafficking, it was necessary to attain near stoichiometric occupancy of newly synthesized class II dimers with CLIP or alternative ligands. We first determined if this could be accomplished by biosynthetic attachment of peptide sequences to the NH_2 terminus of the β chain (30) (Fig. 1). Earlier studies with such engineered β chains showed high binding site occupancy of those class II molecules reaching the cell surface (21) but did not examine whether the peptide was inserted in the ER or later after movement to other intracellular organelles, nor the efficiency of transport of these molecules. A protein containing the Eα52-68 peptide associated with the $A\beta^b$ chain [Eα52-68:$A\beta^b$] was used, because binding of free Eα52-68 peptide to A^b produces class II dimers that remain intact during SDS-PAGE, and the mAb Y-Ae recognizes A^b dimers only when they have this peptide in the binding groove (27), providing two measures of effective occupancy. In transfected COS cells metabolically labeled for 40 min, a substantial amount of Y-Ae–precipitable material was present that ran as a stable dimer in SDS-PAGE (Fig. 2 *A*), indicating that these class II molecules had the tethered Eα peptide sequence properly bound in the groove. The endo H sensitivity of the N-linked glycans on the α chains of these molecules

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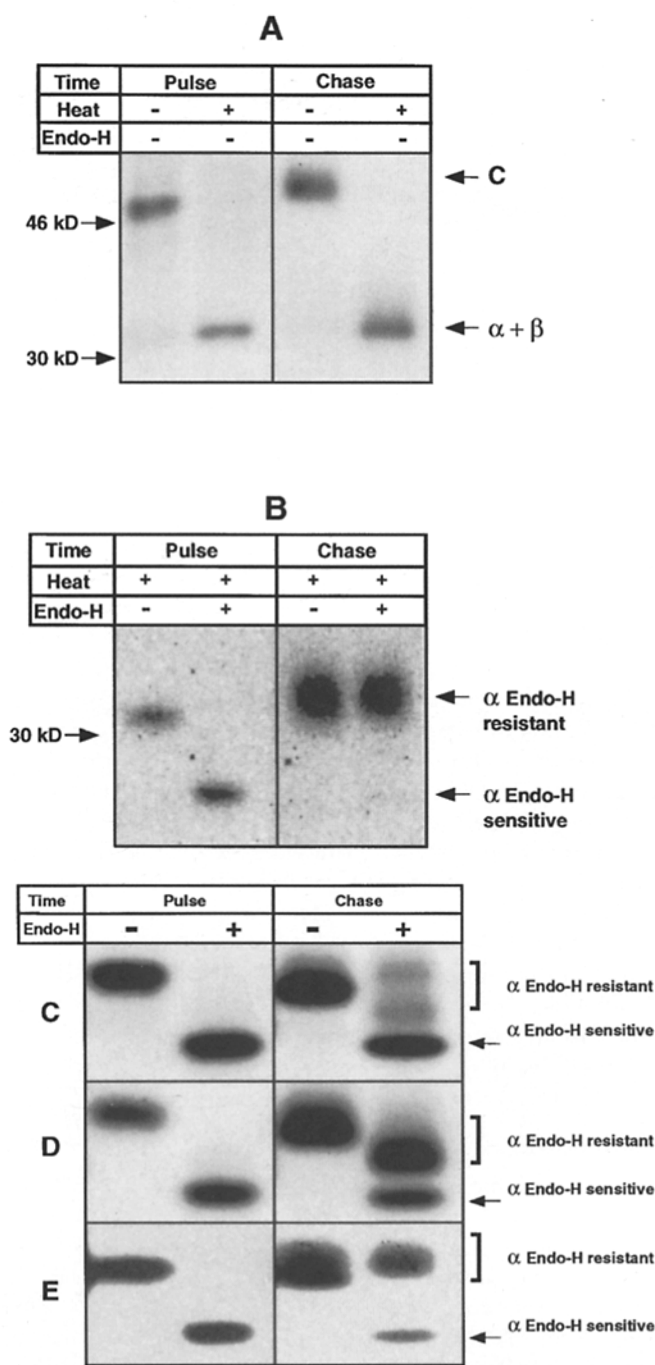

Figure 2. ER occupancy of the class II binding site by tethered peptides promotes rapid transport of these loaded molecules through the Golgi complex and substitutes for the function of Ii. (*A*) Aβ^b chains tethered to Eα52-68 form SDS-stable dimers with wild-type Aα^b in transfected COS cells. Class II dimers were precipitated from pulse-labeled, transfected COS cells and subjected to SDS-PAGE without prior sample heating (*left lane*) or with sample heating (*right lane*). *C*, compact, SDS-stable dimers. (*B*) Binding of tethered Eα52-68 to the binding groove of class II occurs before transport to the Golgi complex. Class II dimers from transfected COS cells were labeled for 40 min, and then isolated α chains were examined to determine the extent of N-linked carbohydrate maturation as a measure of transport through the Golgi complex. (*C*) Wild-type Aα^b and Aβ^b coexpressed without Ii in COS cells show only poor movement from the ER to the Golgi complex Class II dimers were precipitated from pulse-labeled or pulse-labeled and chased transfected COS cells coexpressing wild-type Aα^b and Aβ^b chains, and the reisolated α chain was examined for endo H sensitivity of the N-linked glycans. (*D*) Wild-type Aα^b and Aβ^b coexpressed with a CLIP-containing membrane-anchored fragment of Ii in COS cells show efficient movement from the ER to the Golgi complex. Cells coexpressing wild-type Aα^b and Aβ^b chains together with the Ii19-107 fragment were treated and the class II proteins were analyzed as in *C*. (*E*) Wild-type Aα^b coexpressed with Eα52-68:Aβ^b form class II dimers occupied with the tethered peptide that show as efficient transport from the ER to the Golgi complex as wild-type A^b dimers bound to CLIP-containing Ii. Cells coexpressing Eα52-68:Aβ^b and wild-type Aα^b were treated and the class II proteins were analyzed as in *C*.

showed that peptide binding involved molecules resident in the ER (Fig. 2 *B*). Thus, this approach provided a method for evaluating whether rapid binding site occupancy mimicking class II association with Ii via CLIP promotes efficient ER-to-Golgi complex trafficking.

Antigenic Peptide Binding Replaces CLIP/Ii Function in Class II ER-to-Golgi Complex Transport. COS cells were then transfected with constructs encoding wild-type Aβ^b and Aα^b chains alone, these wild-type chains together with the minimal Ii segment (Ii19-107) able to provide CLIP-dependent enhancement of secretory pathway trafficking (13), or the Eα52-68:Aβ^b chain plus Aα^b. Cells were pulse labeled or pulsed and chased for 4 h, and then the class II proteins were analyzed for SDS stability and the endo H sensitivity of α chain glycans. In agreement with previous studies (8, 9), none of the wild-type class II molecules expressed with or without Ii19-107 had the SDS-stable phenotype associated with tight peptide binding (data not shown). All pulse-labeled samples showed only endo H–sensitive carbohydrates on the α chains, consistent with their residence in the ER (Fig. 2, *C–E*). After 4 h of chase, only a minor fraction (<20%) of the dimers produced in the absence of Ii contained Aα chains with N-linked glycans resistant to endo H digestion (Fig. 2 *C*). Coexpression of the 19-107 fragment of Ii markedly enhanced the transport of these wild-type class II dimers, with 70% of the Aα chains showing evidence of Golgi passage after 4 h (Fig. 2 *D*). In contrast to the wild-type A^b molecules, 75% of class II molecules containing the Eα peptide-linked β chains were SDS stable after pulse labeling (data not shown) and strikingly, the same proportion showed evidence of passage to the Golgi after 4 h (Fig. 2 *E*). This agrees with the data obtained using the Y-Ae antibody, which showed that all of the loaded molecules detected with this reagent transported efficiently (Fig. 2 *A*). Thus, a tethered peptide was as effective in promoting class II transport as was CLIP-containing Ii.

MHC class II α chains have two N-linked glycans. In normal hematopoietic cells, one glycan is typically fully processed to the mature form, whereas a variable amount of the second becomes endo H resistant. The differences in the pattern of endo H–resistant forms for the α chain in Fig. 2, *C–E*, indicate that the interaction of class II with large ligands such as Ii or perhaps large proteins, as compared to short groove-filling peptides, alters the ratio of such glycan processing.

To determine if this capacity of binding site occupancy to enhance transport was a general property of class II

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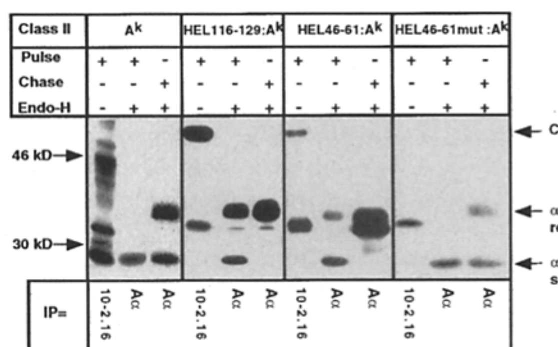

Figure 3. Effective peptide occupancy of the binding region of newly synthesized class II A^k molecules is required to promote efficient transport from the ER through the Golgi complex. RBL transfectants expressing the indicated wild-type or peptide-associated class II proteins were pulse labeled or pulse labeled and chased. Class II dimers were immunoprecipitated and either analyzed for SDS stability or for the endo H sensitivity of the α chain N-linked glycans.

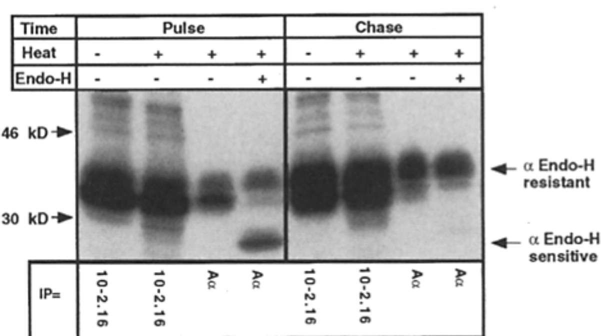

Figure 4. The CLIP sequence tethered to A^k molecules replaces Ii function for class II ER-to-Golgi complex transport without inducing formation of SDS-stable dimers. RBL cells were cotransfected with constructs encoding wild-type $A\alpha^k$ and $A\beta^k$ tethered to the sequence of mouse CLIP. Cells were labeled and class II proteins were analyzed as in Figs. 1–3.

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dimers, we conducted similar experiments using tethered peptides associated with the $A\beta^k$ chain and expressed in a distinct cell type. A^k has a very low affinity for CLIP (31), in contrast to A^b its assembly is not noticeably affected by Ii (9), and it is less dependent on Ii for transport than A^b (32). Wild-type A^k chains, as well as $A\beta^k$ chains attached to HEL peptides 46-61 or 116-129 coexpressed with wild-type $A\alpha^k$, were analyzed as described above for A^b. Wild-type A^k formed SDS-unstable dimers in the ER that showed no Golgi complex trafficking during the pulse–labeling period, whereas 65% of the molecules had endo H–resistant glycans after 4 h of chase (Fig. 3). In contrast, A^k dimers bound to the HEL 46-61 or 116-129 sequences were largely SDS stable in the ER, and after only 40 min, ~20% of the molecules already showed evidence of Golgi complex transit. After a 4 h chase, essentially all the molecules had endo H–resistant glycans (Fig. 3). Thus, the ability of covalently attached peptide sequences to promote class II transport appears to be a general property of these ligands.

The spacer between the tethered peptide and the beginning of the authentic Aβ chain lies near one of the Aα chain carbohydrate attachment sites. Because N-linked glycans are involved in calnexin binding (33), the transport-promoting properties of these constructs might have related not to binding site occupancy, but to steric interference with calnexin binding and loss of ER retention. To examine this issue, multiple residues in the 46-61 peptide, including those involved in binding to A^k, were mutated. Class II molecules tethered to this nonbinding sequence failed to show enhanced trafficking from ER to Golgi complex, although the β chains still had the peptide linker region attached (Fig. 3). This provides strong evidence that trafficking is dependent on binding site occupancy.

CLIP Alone Replaces Ii Function in Class II ER-to-Golgi Complex Transport. In all these cases using tethered antigenic peptides, most class II molecules rapidly folded into compact, SDS-stable dimers, and transport was quantitatively related to such stable dimer formation. Ii–class II complexes, however, do not have this SDS-stable conformation (14). To examine whether the SDS-stable conformation was essential to transport, and to test whether CLIP alone could replace Ii function, a construct encoding the core region of mouse CLIP tethered to $A\beta^k$ was used. Coexpression of this chain with $A\alpha^k$ did not lead to generation of SDS-stable dimers (Fig. 4), consistent with other studies showing that many ligands do not produce SDS-stable forms when they associate with the binding domain of class II (29, 34, 35). Nevertheless, the CLIP-containing complex still transported more efficiently than wild-type A^k dimers, indicating that the CLIP segment possesses by itself all the properties necessary for enhancing class II ER-to-Golgi complex transport, and further, that it can mediate this effect without inducing the SDS-stable conformation of class II.

Discussion

Although the class II chaperone function of Ii is well appreciated, it is still widely held that Ii interacts with the class II binding groove via CLIP to inhibit presentation of endogenous peptides intended for class I binding in the ER. Given the present data, the absence of SDS-stable dimers in the ER of the cells of Ii-deficient mice and the failure of SDS-unstable class II dimers in such cells to transport effectively clearly indicate that most class II molecules do not associate tightly with peptide ligands in that organelle even when Ii is totally absent (8). Our observations thus support the alternative hypothesis that the primary secretory pathway functions of Ii, and of CLIP in particular, are to stabilize properly folded class II dimers and facilitate their secretory pathway trafficking in a functional state (1, 18, 19).

All tested short ligands capable of effective interaction with the class II binding region promoted secretory pathway transport of class II. This was true both for antigenic peptides that led to class II folding into a highly stable dimer conformation and for CLIP itself, which facilitated

such movement with class II remaining in the SDS-unstable state characteristic of class II–Ii complexes (14). This peptide-mediated class II transport represents one of the few examples of ligand-dependent trafficking of receptor proteins in the secretory pathway (36). The ease of manipulating the involved peptide together with knowledge of the detailed structure of class II molecules make this an intriguing model for studies relating protein folding to exit from the ER.

It thus seems that class II molecules have evolved to be highly dependent on ligand binding for their proper intracellular movement and functional survival under physiologic conditions, as originally observed for class I molecules (37). Some form of binding site engagement must be provided shortly after class II biosynthesis to prevent loss of activity during transport to endocytic locations, and CLIP association with the groove fulfills this requirement. Concerted exchange of CLIP for antigenic peptides via the action of DM (38–40) also conforms to the idea that the class II binding site cannot be left unoccupied other than transiently, as does inactivation of surface-expressed class II molecules after loss of peptide, unless a new ligand is quickly acquired (14–16). Thus, for its optimal functioning, a class II molecule must have its binding site properly chaperoned at all stages of its post-synthetic lifetime.

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Address correspondence to Ronald N. Germain, Laboratory of Immunology, Building 10, Room 11N31, 10 Center Drive, MSC-1892, National Institutes of Health, Bethesda, MD 20892-1892.

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